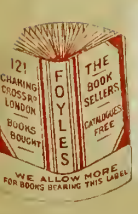
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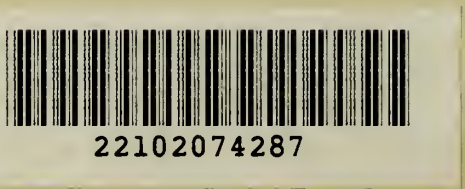
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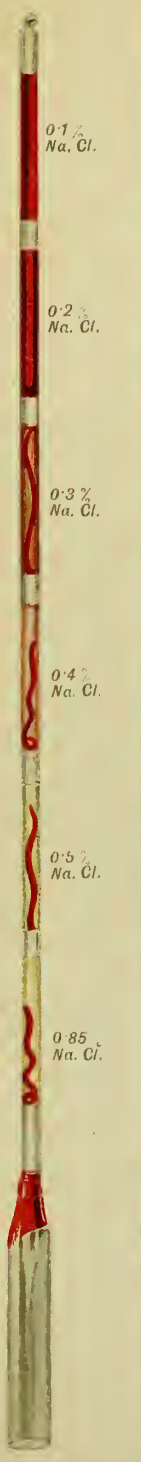

Fig 1

Fig 2

Fig. 3.

Fig. 4

Frontispiece

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FRONTISPIECE.

Fig. 1. Composite pipette throttled at the proximal end and filled in with a series of test mixtures consisting in each case of one unit volume of blood mixed with two unit volumes of diluting fluid, the salt content of the diluting fluid diminishing in the test mixtures towards the distal end of the tube. In relation with this we have, in the proximal test mixture, a scarlet blood clot containing a full quantum of unlaked red corpuscles, and a hæmoglobin-free serum; in the more distal test mixtures more and more attenuated and more crimson coloured clots are sinking down into the sera which are more and more deeply stained with hæmoglobin; and finally, in the most distal test mixture, we have a clot whose meshes enclose a mere débris of hæmolysed corpuscles which is so light as to allow the clot to remain suspended in the lake-coloured serum (vide *Chapter VII., Appendix*).

Fig. 2. A similar throttled pipette filled in with a series of test mixtures containing one unit volume of blood mixed in each case with one unit volume of oxalate of ammonium solution, which is more and more diluted towards the proximal end of the tube. In relation with the progressive decalcification, which is thus brought about, is the fact that we have in the proximal test mixture a practically normal clot—no corpuscles lying loose—and a layer of serum intervening between the clot and the sides of the tube; and in the more distal test mixtures progressively smaller clots and more and more corpuscles lying loose; until in the last three test mixtures we have no clot and all the corpuscles lying loose giving us a supernatant layer of plasma, and below this a layer of corpuscles (vide *Chapter VII., Sub-section* 14).

Fig. 3. A hæmocrit pipette with inserted throttle upon the stem of which are inscribed six divisions corresponding each to a unit volume, and a division corresponding to twelve unit volumes. The pipette has been filled in with ten volumes of blood and two of citrate of soda, and the settlement has taken place giving us approximately 55 volumes per cent. of corpuscles (*Chapter VII., Sub-section* 2).

Fig. 4. Throttled pipettes filled in each case with a series of mixtures consisting of one unit volume of a suspension of typhoid bacilli mixed with progressively increasing dilutions of serum derived from a typhoid patient (test mixtures in distal portion of tube) or a unit volume of physiological salt solution (control mixture in proximal end of tube). In *A* are shown the phenomena of agglutination as seen very shortly after the tube has been filled in.

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In the 128 dilution of the serum is seen an *incomplete agglutination effect*, and a certain number of bacteria which have not been agglutinated have been brought down by gravitation upon the top of the agglutinated bacterial deposit.

C shows the effect of turning the tube upside down. In the control mixture there is here obtained a very fine rain of separate bacterial elements; in the test mixture a hail of agglutinated bacterial masses.

HANDBOOK OF THE TECHNIQUE OF THE TEAT AND CAPILLARY GLASS TUBE

AND ITS APPLICATIONS IN MEDICINE AND BACTERIOLOGY

BY

SIR A. E. WRIGHT, M.D., F.R.S.

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"DIE METHODE IST ALLES."—*Carl Ludwig*

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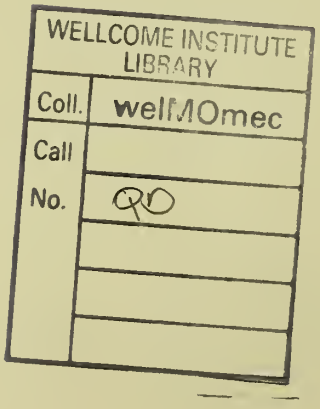

PREFACE.

The system of technique—I call it the *technique of the teat and the capillary glass tube*—and almost all the quantitative methods of blood examination which this book teaches are affiliated to a procedure described by me in 1897 in a paper on Widal's test for typhoid fever.

I pointed out there that by using a mark placed anywhere on the stem of a simple pipette, and an air-bubble index, we can make any required dilution of the serum, mix it in accurately measured quantities with the culture, and then with the naked eye read off the result in the pipette.

Very soon afterwards the use of rubber teats for operating the pipettes suggested itself, and the technique was now complete in its essentials. It will be appreciated that what had here come to birth was something more than a procedure for measuring a single function of the blood. It was a general technique for conducting quantitative tests in uncalibrated capillary tubes with minimal quantities of reagents. It was now possible, as soon as the need for these should make itself felt, to elaborate methods for measuring also other functions of the blood. The need made itself felt first in connection with anti-typhoid inoculation; then in connection with the investigation of scurvy, hæmophilia, chilblains, and urticaria, and the treatment of these; and finally and most urgently in connection with vaccine-therapy.

Under such impulsion the methods which are set forth in the body of this book evolved themselves. And, again, out of these there developed a more or less abstract system of technique—the technique proper of the teat and capillary tube—which will, I think, in the future find wider application.

In conformity with this differentiation of its subject matter, this book makes appeal to two different classes of laboratory workers: to the man who wants a ready-made technique for measuring this or that function of the blood, and wants nothing more; and to the research worker who looks not only to applications of the technique already made, but to the further applications which will follow now that science is flooding everywhere into medicine.

I have here tried to furnish the former class of worker with a training school in the technique, and the research worker with a critical text book.

From explaining what this book is in intent, I pass to consider the question—it will come up in the mind of everyone who sees or tries the technique for the first time—as to whether this technique demands from the man who undertakes it too much manipulative skill.

If it did, there would not be anything to say in its defence.

For it is the very purpose and *raison d'être* of technique to eke out the inadequate skill of our hands by the contributory work of our brain. It will accordingly be only bad technique which requisitions exceptional manipulative dexterity. And the characteristic of good technique will be that it brings feats which look impossibly difficult within the compass of every man's capacity.

But, as our definition of technique teaches, something must be supplied to take the place of manual dexterity. There will be need for intellectual labour, for forethought, for a certain gift of concentration, and for a reasonable amount of practice.

All this has direct application to the question here before us. The manipulations in which the newcomer sees special dexterity are in reality within the capacity of anyone who will bring to the task a little intelligence and a reasonable modicum of application and practice.

On the question as to what would constitute that modicum, different minds will go frankly apart.

One kind of ridiculous person will demand that the technique should come to him immediately and by the light of nature; a *second* will come to the laboratory and ask to be taught the technique while he keeps his wife waiting outside in a taximeter cab; and a *third* will spend "*two* headachy afternoons" in trying to teach himself the technique, and will be indignant at want of success after such effort.

But there will also be another kind of man. He will call to mind that no one can hope to acquire proficiency in billiards or golf unless he gives up years to the task. He will reflect also upon those words of Ludwig which proclaim that "the whole of science lies in technique." And he will be quite prepared to learn that it will, after he has mastered all the ordinary laboratory technique, take him several weeks of concentrated labour to possess himself of the methods here described, and to

"catch
Hints of the proper craft, tricks of the tool's true play."

The conclusion of a task which gathers together the work of years inevitably brings back that past. With it there comes to me a

realisation of all the help which my present and past fellow-workers have given in the elaboration and perfecting of the technique. While I have not neglected to make such acknowledgments where opportunity offered, I am conscious that their suggestions and contributions must often have become merged into the body of technique and have been forgotten. To two, however, of my fellow-workers I owe quite special and unforgettable acknowledgments: to Sir W. B. Leishman, for the origination of the idea of measuring the phagocytic power of the blood, and for the first practical working out of that idea; and to Capt. S. R. Douglas for working out in association with me—and it was a very arduous task—the technique for measuring the opsonic power of the blood.

In connexion with the preparation of the present treatise, I am indebted to my fellow-worker, Dr. W. E. M. Armstrong, for making a first draft of portions of the text, for reading the proofs, for the preparation of the index, and generally for valuable help and criticism.

I owe also very grateful acknowledgments to my artist, Mr. J. H. Wybrants, for the skill and intelligent comprehension which he has brought to bear upon the task of illustrating the book.

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PLATE I.

Fig. 1 *a* and *b* show the conditions under which bubbles are formed when reaspirating fluid into the pipette. *Chapter IV., Sub-section* 6*b*.

Fig. 2 (*a*) shows the conditions under which the aspiration of bubbles from a drop of fluid into the pipette occurs.

(*b*) shows how such aspiration may be prevented. *Chapter IV., Sub-section* 6*b*.

Fig. 3 (*a*) shows the way in which—on the one hand—the formation of bubbles on blowing out the pipette, and—on the other hand—the reaspiration of fluid into the tube, may be prevented. *Chapter IV., Sub-sections* 6*a* and 7.

(*b*) and (*c*) show how when the end of the pipette is shorn off at a slant the fluid does *not* disengage itself from the orifice, and so gets blown out into bubbles. *Chapter IV., Sub-section* 6*a*.

PLATE II.

Fig. 1. A blood capsule containing blood showing hæmagglutination.

a. The blood as it appears a few minutes after the capsule has been filled in. The blood has here differentiated itself into a corpuscular deposit and a milky supernatant plasma. The agglutination of the red blood corpuscles is indicated to the eye by the rapid sedimentation, the granular appearance, and irregularity of the delimiting contour of the corpuscular deposit. The milkiness of the plasma is due to the presence of innumerable blood platelets (*Chapter V., Sub-section* 9).

b. The same blood as it appears a few hours later. The fibrin meshwork which has formed in the corpuscular deposit and plasma has here contracted, drawing together the former, and collecting together out of the latter all the suspended particles into a "buffy" coat. In association with this a water-clear serum has separated itself out.

Fig. 2. Blood capsules showing blood from which a milky serum separates out (*Chapter V., Sub-section* 8).

a. Blood as it appears a few minutes after filling in the capsule.

b. The blood as it appears a few hours afterwards. It will be noted that there is here no "buffy" coat and that the serum is still milky. The milkiness is thus due not as in Fig. I. (*a*) to comparatively gross suspended particles, but to elements of almost molecular fineness (*Chapter V., Sub-section* 8).

Figs. 3 and 4 show the results obtained in the Wassermann test (*Chapter X., Sub-section* 9).

Fig. 3 (*a*). Tubule containing a normal serum mixed with sheep's corpuscles—hæmolysis.

Fig. 3 (*b*). Tubule containing normal serum which has been digested with a differentiating antigen, receiving afterwards an addition of sheep's corpuscles —hæmolysis.

Fig. 4 (*a*). Tubule containing a syphilitic serum mixed with sheep's corpuscles—hæmolysis.

Fig. 4 (*b*). Tubule containing a syphilitic serum which has been digested with the same differentiating antigen, afterwards receiving an addition of sheep's corpuscles—no hæmolysis.

Fig. 5. Method of making a series of dilutions which stand to each other in the same relation as the terms in a geometrical progression (*Chapter IV., Sub-section* 13).

(*a*) shows a series of drops of diluting fluid each corresponding to one unit volume, or to two or more unit-volumes.

(*b*) shows the series of mixtures obtained by adding to the first drop a unit volume of the dilutand (here blood), and carrying on in each case one unit-volume of the mixture from the preceding drop to the next in series.

Fig. 6 shows the appearances obtained when the blood of a leucæmic patient is allowed to settle down by gravitation after decalcification by admixture with citrate of soda (*Chapter V., Sub-section* 6).

Fig. 7 shows the appearances obtained when in connexion with the measurement of the coagulation-time capillary tubes which have been filled in with blood are blown out into water (*Chapter VII., Sub-section* 9).

a. Here coagulation has not commenced and the red corpuscles, when projected from the tube, separate out from one another forming a cloud which melts away as the corpuscles dissolve in the water.

b. Here coagulation has just commenced, and the corpuscles are collected and held together into clumps by the fine meshwork of fibrin which is beginning to form.

c. Here coagulation is complete, and a firm snake-like clot has been obtained.

d. Here a snake-like clot such as was obtained in (*c*) has undergone hæmolysis, and it now contains only the leucocytes of the blood and shadows of red corpuscles (*Chapter VII., Sub-section* 20).

PLATE III.

This plate shows what has to be avoided and what is to be aimed at in making film-preparations of blood, and in particular film-preparations for phagocytic counts.

a. An extreme example of the unsatisfactory honeycomb film which is obtained when the surface tension of the blood fluid strips it off from some region of the slide leaving the glass bare, and piles it up on other regions until the corpuscles lie many layers deep.

b. An example of a film with a concave end. Such a film is produced when the spreader is held too upright or when its working end is too much hollowed out.

c. An example of a film drawn out into a tongue by a rectangular spreader which has also been held too obliquely.

d. Example of a film which is open to criticism only in the respect that it is carried a little too far down the slide.

f and *g*. Examples of unsatisfactory films.

The frilled edges and striation marks which are the characteristic features of these films show that the slide which was used for spreading them had an edge toothed like a comb, and that a heavy hand brought it down upon the film, almost stripping the slide in some places, and here lacerating the leucocytes; and piling up the blood thus collected into ridges in which the leucocytes would retain their spherical shape instead of being flattened out.

These films were made by the Author of a paper on the "Inaccuracy of the Opsonic Method," and were put forward by him as satisfactory.

e. Example of a satisfactory film.

Made by the same Author after he had allowed himself to be taught.

h. A quite satisfactory film.

PLATE IV. illustrates the method of measuring the bactericidal power of the blood. (*Chapter* VIII., *Section* II.).

a. Looped pipette which is being filled in with litmus mannite broth which will afterwards tell us whether the blood has exerted a bactericidal effect on the typhoid bacillus.

b. The nutrient fluid has been carried up into the cultivation chamber and one unit volume of serum and one unit volume of a dilution of the typhoid culture have been measured into the stem of the pipette.

c. The serum and the dilution of the typhoid culture have been mixed and blown out, and have now been reaspirated into the stem, and the end of this has been sealed in the flame. The teat has been withdrawn and the dilution of the culture has been inscribed on the mouthpiece of the pipette.

d. After 24 hours in the incubator the test-mixture is being drawn up into the incubation chamber. To this end the capillary stem has been throttled, and the teat has been reimposed on the mouthpiece of the pipette in the collapsed condition.

e. The pipettes have been returned to the incubator for 24 hours in order to ascertain whether the typhoid bacilli have, or have not, been killed off by the action of the serum.

In the case of the particular dilution of the culture which was employed in this pipette they have been completely killed off. This is shown by the fact that the litmus mannite broth has remained clear and unchanged in colour.

f. In the case of the particular dilution of the culture which was employed in this pipette the typhoid bacilli have not all been killed off. This is evidenced by the change of colour and the slight turbidity and deposit in the nutrient medium. The change of colour is explained by the fact that typhoid bacilli produce acid when grown in litmus mannite broth.

PLATE V.

Fig. 1. The edge of an opsonic film (under a comparatively low power), showing the leucocytes gathered to the end. *Stained with carbol thionin.*

Fig. 2. A portion of the edge of an opsonic film (under a high power), showing phagocytosis of staphylococci. *Stained with carbol thionin.*

Fig. 3. A portion of the edge of an opsonic film under a high power, showing phagocytosis of tubercle bacilli. *Stained with carbol fuchsin and methylene blue.*

Fig. 4. A zoogloeic mass from a tubercle culture (under a high power).

Fig. 5. Enumeration of bacteria in a vaccine. A portion of the preparation under a very high magnifying power.

CHAPTER I.

DESCRIPTION OF THE APPARATUS AND MATERIALS WHICH OUGHT TO BE AT THE DISPOSAL OF THE LABORATORY WORKER WHO PROPOSES TO MAKE USE OF THE METHODS DESCRIBED BELOW.

Glass working table—Glass-tubing—Coloured glass-writing pencils—Rubber teats—Mercury—Standardised pipettes—Plasticine—Opsonic incubator (opsoniser)—Ball and socket pestle and mortar—Centrifuge—Emery paper—Staining rack.

1. Glass working table.

Any ordinary glass working table provided with a foot bellows of sufficient size will serve.

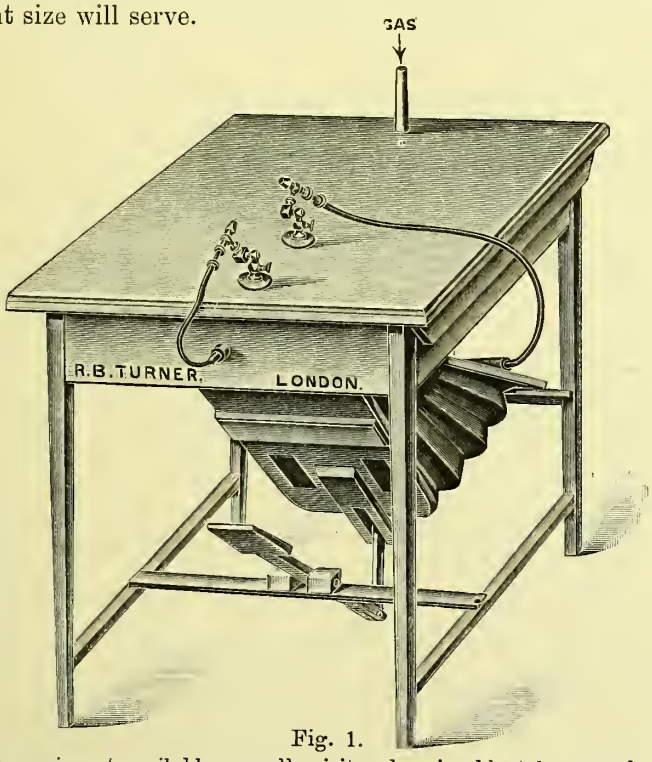


Fig. 1.

Where gas is not available a small spirit or benzine blast lamp such as is used by braziers will have to do duty instead.

2. Glass-tubing.

The dimensions of the glass-tubing required will be as follows:— For the manufacture of capillary pipettes and the smaller apparatus generally, tubing of about 5 mm. external diameter and with walls a little less than 1 mm. thick; for the manufacture of larger apparatus —such as *tubules* and *centrifuge tubes* respectively—tubing having an external diameter of 6 to 10 mm. with walls approximately 1·5 mm. thick.

Where we require tubing of wider calibre with comparatively thin walls we may conveniently make use of stout walled test-tubes.

Fig. 2.

The quality of the glass which is employed is of great importance, quite unnecessary difficulties being introduced into glass working operations by the use of inferior qualities of glass. Glass which becomes opaque and brittle or which "runs" on heating—glass which would unhesitatingly be rejected by the professional glass worker—is often offered for sale for the use of laboratory workers. All such glass, and also all glass with unduly thin walls, and all lead glass—for this blackens in the reducing flame —is to be rejected. What is required for our purposes is a hard soda glass which becomes plastic without running when heated in the blow-pipe flame.

The question of alkalinity must also be considered in connexion with glass. Many of the cheaper kinds undergo, in particular when heated in the blow-pipe flame, a disintegrative change which goes hand in hand with the setting free of alkali. Where such change has occurred distilled water will be found to take up from even a capillary tube sufficient alkali to turn red litmus paper distinctly blue. Glass of this kind must be discarded as introducing a possible source of fallacy into our experiments and observations. The matter acquires special importance in connexion with observations upon the alkalinity of the blood.

3. Coloured glass-writing pencils.

These differ from ordinary coloured pencils in the respect that grease is incorporated into the crayon with a view to marking the smooth surface of glass. In cold weather, owing to the congelation of the grease the glass surface upon which we write must be gently warmed by friction, or by passing it—once, very rapidly—through the flame.

The dark blue pencils are the best for ordinary purposes, as they are less brittle and can be brought to a finer point.

The marks made by these pencils can be fixed upon the glass by heating them in flame until the grease begins to char and to turn black.*

4. Rubber teats.

For the purposes of the ordinary technique what are required are teats made from the best soft vulcanised rubber having a capacity of about 1·5 ccs. Larger ones having a capacity of 2·5 ccs. are only very exceptionally required.

Fig. 3.

It is absolutely essential to the accurate working of the technique that the teats should always fit air-tight upon the tubes. The devices which can be resorted to when a leak develops at the joint are explained in *Chapter IV., Sub-section* 3.

Perforated teat.—Where a teat is to be imposed upon a capillary pipette in such a manner as not to alter the air pressure in its interior (*vide Chapter IV., Sub-section* 8, *b.*), we may conveniently employ a perforated teat—made by burning a small hole with the fused end of a capillary tube into the neck of the teat just above the collar.

Fig. 4.

Throttled teat.—In practically every case where in connexion with the use of an ordinary teat the throttling of a capillary tube is enjoined, we may, as suggested by my late fellow-worker, Prof. B. J. Collingwood, employ instead a throttled teat. The throttled teat is made by taking a piece of glass tubing which will just pass up comfortably into the mouth of our teat; drawing it out a little in the middle; cutting it down on either side of the constriction till it measures about 1·5 cm.; luting into it with sealing wax as shown in the figure a throttle made from a tube of finer bore; passing this little piece of apparatus into the upper part of the neck of the teat; and fixing it in position there by an ordinary ligature or a couple of turns of fine wire.

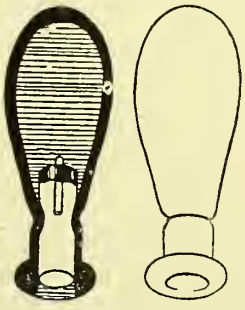


Fig. 5.

* It may be noted that the charring of these pencil-marks—a charring which is particularly conspicuous in red-pencil-marks—supplies us with a convenient thermometer. If, where we are sterilising a test-tube, a vaccine-bulb, a tubule, a watch-glass, or any other piece of apparatus by passing it through the flame, we make a mark or marks upon it with a red glass-writing pencil, and then heat until these turn black, a temperature of over 200° (C.) will have been reached, and we may be sure that our piece of apparatus is sterile.

5. Mercury.

In connexion with the graduation of capillary pipettes a small quantity of mercury will be required.

When, as will in process of time inevitably happen, the mercury has picked up chemical impurities and adheres to the glass instead of flowing smoothly and holding together by means of its surface tension, it may be purified by stirring it into strong nitric acid, and then washing thoroughly with water.

When the mercury has become dusty, the particles of dust may be filtered out by pressing the mercury through fine linen, or, if such is to hand, through a piece of chamois leather; or by letting the mercury drip through a funnel made by drawing out a piece of wide glass-tubing into a very fine throttle.

6. Standardised pipettes.

Where we are making volumetrical measurements and require a set of standards, we may conveniently provide ourselves with a series of standard pipettes. Such pipettes, bearing the Charlottenburg hall-mark, can be procured through any laboratory purveyor.

7. Plasticine.

This is a patented material which possesses physical properties intermediate between putty and modelling wax, and which is free from the defects of these materials, inasmuch as it is plastic at all ordinary temperatures, does not leave a greasy stain, and retains its plasticity indefinitely. It finds many applications in connexion with the technique described below.

In England it can be purchased at laboratory purveyors, toyshops, and shops which supply artists' materials.

In connexion with the measurement of the opsonic power of the blood the following additional apparatus is all but indispensable.

8. Opsonic incubator (opsoniser).

While an ordinary bacteriological incubator, or in fact any appliance which will maintain a temperature approximating to blood-heat for a reasonable time, will serve for an occasional measurement of the opsonic power of blood, it is very desirable where such measurements are carried out as a matter of routine to have an incubator which is specially designed for this work.

That which was introduced by my fellow-worker, Dr. J. Freeman, is both simple and satisfactory (Fig. 6). It may be well to explain that the tubes whose numbered openings are seen on the front face of the incubator go right through to the back, and that each accommodates a single capillary pipette.

The essentially important feature in this type of opsoniser is a good regulator, for the regulation of the temperature is made difficult by the fact that the apparatus holds comparatively little water while presenting an extensive surface area to the air.

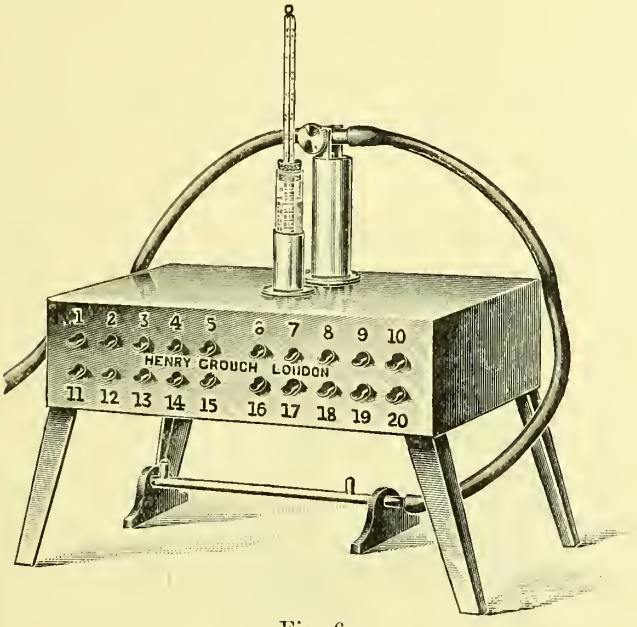


Fig. 6.

A wind screen may also with advantage be fitted to protect the flame against air-currents.

Where in fitting out a laboratory economy has to be studied it is possible to adapt the ordinary type of bacteriological incubator for opsonic work by perforating the water chamber by a set of tubes, and such incubators could of course easily be made to order.

As suggested, among others, by Dr. Reiter, a thermos bottle with holes perforated in the lid may be employed as an opsoniser. It is, however, in the long run, inconvenient to have one's pipettes disposed vertically instead of horizontally.

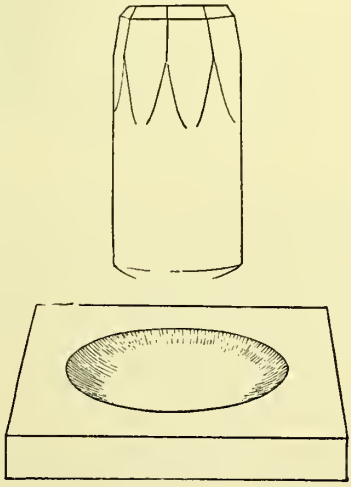


Fig. 7.

9. Ball and socket pestle and mortar.

This pestle and mortar finds application when we have to break up

bacterial agglomerations, such as those with which we have to deal in tubercle cultures, into separate elements for the preparation of a bacterial suspension. The characteristic feature of the design—and this was suggested by my fellow-worker, Captain A. F. Hayden—is that the convexity of the pestle has been ground to fit the concavity of the mortar, after the manner of a shallow ball and socket joint. The pestle and mortar are made of glass. Both labour and material are economised by its use. It can be obtained from Messrs. Angus, of Wigmore Street, London, W.

10. Centrifuge.

A very efficient centrifuge will be required for the preparation of the washed corpuscles and for the preparation of satisfactory bacterial

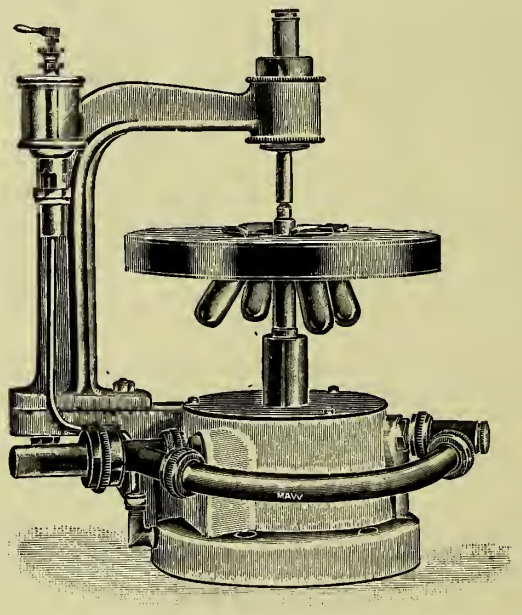


Fig. 8.

suspensions. By far the best pattern of centrifuge for our purpose is that designed by Dr. C. J. Martin, of the Lister Institute. It works very silently, does not require any protecting cover, and is so simple in construction that it requires no attention beyond occasional oiling. As usually sent out it is driven by a water-turbine which is directly attached to the shaft, the water being supplied either by one or two jets.

Where the necessary water pressure cannot be obtained—and a minimum pressure of 30-lbs. to the square inch is required for its

efficient working—the centrifuge can be geared up with a free-wheel pulley to an $\frac{1}{10}$th h.p. electric motor.

In England, Martin's centrifuges can be obtained from any purveyor of laboratory apparatus, such as Messrs. Maw & Son, of Aldersgate Street, E.C., Messrs. Baird & Tatlock, of Cross Street, Hatton Garden, E.C., or Mr. R. B. Turner, of 11, Foster Lane, Post Office, E.C.

Where neither water nor electrical power is available it will be necessary to fall back upon hand centrifuges. In connexion with these it is important to note that machines with heavy-running parts which are capable of storing enough momentum to keep them running

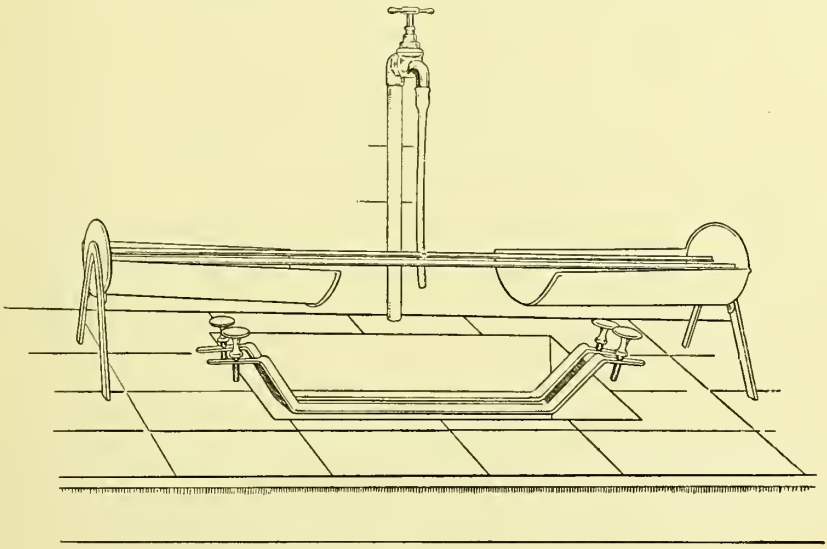


Fig. 9.

steadily for many minutes after the application of force has been discontinued, are in every way preferable to the ordinary light type of machine where the power has to be applied continuously.

11. Emery paper.

Emery paper is required for roughening the surfaces of slides preparatory to the spreading of blood-films upon them. The coarse variety sold for domestic use is quite unsuitable for this purpose. What is required is the fine variety which is used by jewellers, and which is known in the trade as Hubert's 00.

In England it can be procured at any large tool shop.

12. Staining rack.

Where a number of blood-films have to be stained at one and the same time and to be treated in all respects alike, it will

be found very convenient to employ a staining rack. As will be seen from the figure we have a choice between two patterns of staining rack. That which is made in the form of an elevated bridge has the advantage of saving some stooping, and it allows of the flame of a Bunsen being carried underneath the slides. That which is made in the form of a sunk bridge takes up, however, less room on the bench.

CHAPTER II.

METHODS OF GLASS WORKING AND MANIPULATING AND DRAWING OUT GLASS TUBING SO FAR AS THESE ARE REQUIRED FOR PURPOSES OF THE TECHNIQUE HERE IN QUESTION.

Introductory—Method of using the blow-pipe flame—Method of making a glass-cutting knife—Method of cutting across a glass-tube—Method of drawing out glass-tubing—Method of bending a piece of glass-tubing—Method of drawing out a piece of tubing into a capillary stem, and bending this round so as to form a recurved limb—Method of drawing out a piece of tubing into a capillary stem, and bending this round to form the loop of a spiral—Method of welding two pieces of glass-tubing—Method of sealing off one end of a glass-tube—Method of sealing up a test-tube or capsule—Method of throttling a capillary tube—Method of drawing out a capillary tube into a rapidly expanding solid point, such as would furnish a satisfactory pricker for obtaining blood from the finger.

1. Introductory.

Inasmuch as glass-tubing constitutes the raw material out of which practically all the apparatus which is required for the technique here in question will have to be manufactured, and inasmuch as the laboratory worker will have to manufacture it all for himself, it will be well once more to emphasise the importance of using only tubing of good quality. This done, I may say a word about the management of the blow-pipe flame and the making of a glass-cutting knife, and then proceed to give a brief description of the very simple glass working operations which will have to be undertaken.

2. Method of using the blow-pipe flame.

For practically all glass-working operations a gas blow-pipe flame worked by a blast from either a mechanical pump or a foot bellows is indispensable. The point of importance in connexion with the blast is that it shall be delivered not in a series of jerks but in a continuous stream. In the case of a foot-bellows the pressure requisite for the delivery of a continuous blast is to be obtained either by a rapid series of initial foot-strokes, or by constricting for a moment the air outlet from the bellows. The air pressure once obtained is easily maintained by regulating the length and rate of the foot-strokes. All these

adjustments will be facilitated by the employment of a large foot-bellows fitted to a suitable glass blowing table. (*Vide Chapter I., Section* 1.)

In using the blow-pipe flame we employ ordinarily only the most distal portion of the flame, for this is the hottest part. But inasmuch as tubing which is large-sized and thick-walled cracks when suddenly exposed to such extreme heat, we do not introduce such tubing directly into this hottest region, but warm it up first either in the inner portion of the blow-pipe flame or in the heated air beyond its distal extremity; or we may undertake the preliminary heating in the cooler luminous flame which is obtained by shutting off the air-blast.

Whenever in the course of our operations strains have been set up in the glass—and such strains are always set up in welding operations and in bending large glass tubing—the joint or bend must, before it is allowed to cool, be carefully annealed, first in the interior of the blow-pipe flame, and finally in the luminous flame.

3. Method of making a glass-cutting knife.

Ordinary glass-tubing, as we shall see, can be broken across after notching with a triangular file, but a glass-cutting knife does the work better and is indispensable when we have to deal with capillary tubes.

Fig. 10.

It is possible to purchase glass-cutting knives, but, inasmuch as any good quality steel can readily be made to cut glass, it is more convenient to convert one of the blades of one's pocket knife into a glass-cutting blade.

For this purpose we introduce the edge of the selected blade into the hottest part of the blow-pipe flame, *i.e.*, into the outer fringe of the extreme end of the flame, and heat it to a full white heat. Then without a moment's delay we plunge the glowing blade perpendicularly into a vessel of cold water placed ready to hand, and hold it there till it has completely cooled down. This done, we roughen the edge of the blade upon a rough whetstone such as is used for sharpening scythes.

The essential objects to keep in view in these several operations are the following:—(*a*) We must heat the knife edge to the highest possible temperature; (*b*) we must, in order to avoid burning off the carbon upon which the hardness of the steel depends, maintain such temperature only for the shortest possible time; (*c*) we must cool down the steel very rapidly in order to give it a high temper, and in plunging it into the water we must avoid unequal cooling of the two faces of the blade and consequent warping; lastly (*d*) we must convert

the originally smooth edge of the blade into a saw edge which will bite into the glass.

In connexion with the tempering of the blade and the working up of its edge upon the whetstone, the following points may be noted :—

While we must aim at giving the highest possible temper to the knife edge, some of the natural toughness of the steel should be retained in the back of the blade. Where we are operating upon a fairly large blade we need not make special provision for this, for the extreme heat of the flame will have been concentrated upon the edge and will have spared the back of the blade. Where, however, we are operating upon a very small blade, the whole of it may have to be raised to a very high temperature, with the result that it may become very brittle through taking on a very high temper. In such a case it will be well to restore the original toughness to the back of the blade

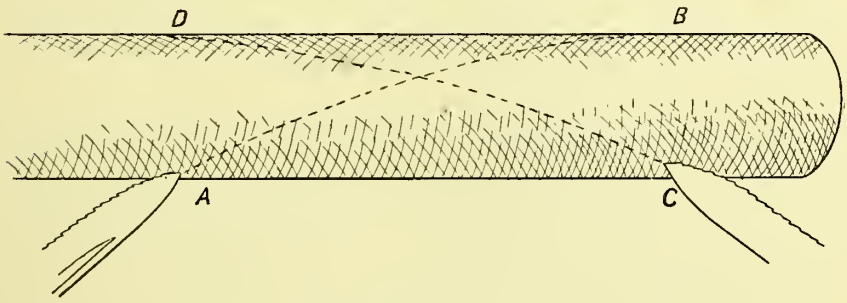


Fig. 11.

by re-heating it in the blow-pipe flame to a dull red heat and then allowing it to cool down slowly in the air.

In the matter of the character of the saw edge it will be well to arrange to cut the notches so that the saw teeth may point backwards in the direction of the handle of the knife, for they will then bite into the glass more effectively. This is simply a question of whetting the blade in a proper manner.

In the outgoing stroke (A to B) the back of the blade should be directed away from the operator and the point should be entered on the waist of the whetstone and be carried obliquely across to the left-hand border of the narrow end of the stone. In the return stroke (C to D) the back of the blade should face the operator and the point should be entered on the right-hand border of the far end of the stone and be carried thence obliquely inwards in such a way that the path of the incoming stroke shall intersect with the path of the outgoing stroke to form an X.

When the knife has become blunt and will no longer cut a capillary tube squarely across, its edge ought to be refreshed upon the whetstone.

The temper of the blade would of course be impaired if it were re-introduced into the blow-pipe flame.

4. Method of cutting across a glass-tube.

Procedure where we are dealing with the ordinary sizes of glass-tubing. —All the ordinary sizes of glass-tubing can be cut by making a small transverse nick with a glass-cutting knife or triangular file and then applying leverage—as shown in the figure—to prise open the crack.

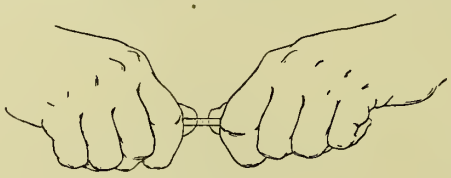


Fig. 12.

If the tubing is of good quality the fracture obtained by these means will be perfectly square and clean, and there will be no occasion whatever to ring round the tube with a circular cut.

The following points may be noted :—

It is the cleanness and the depth, not the breadth and length, of the incision which counts. With an absolutely clean deep linear cut, such as is delivered by a very sharp glass-cutting blade, the tube breaks across at a touch. With a broad interrupted shallow notch, such as is made by a triangular file—and in particular by a worn file —the tube can be broken across only by the exercise of considerable leverage.

When a triangular file is employed it ought to be brought down upon the glass slantwise in the manner shown on the right-hand side of the figure. By using the file thus we not only obtain a better cutting edge, but we avoid the risk of the wedge-shaped file breaking through the tube, and driving the glass into the finger, as it easily may when the edge of the file is brought down, as on the left-hand side of the figure, directly upon the tube, and the glass is thin.

Fig. 13.

Procedure where we are dealing with wide glass-tubing.—Wide glass-tubing—and test-tubes, as well as the larger sizes of ordinary tubing come under this denomination—cannot be simply nicked and broken across. A clean square fracture can here be obtained only by the exercise of a certain amount of skill. The simplest method of procedure—and this procedure is applicable to all tubing except very thin walled test-tubes—is to notch the tube as deeply as possible at the point where it is to be broken across; and then to apply to the notch either, as in Fig. 14, a comparatively small and very hot molten bead of

glass, or better, for this gets down into the notch, a piece of glowing charcoal trimmed to a point. This will, like the mechanical leverage which comes into application in connexion with the smaller sizes of tubing, open up the crack and cause it to run round the tube. It will be recognised that the depth of the incision and the high temperature and restricted dimensions of the source of heat which is applied are essential factors to the success of the procedure.

Another method which is applicable in the case where the lower end of a test-tube is to be amputated is to fill it with cold water up

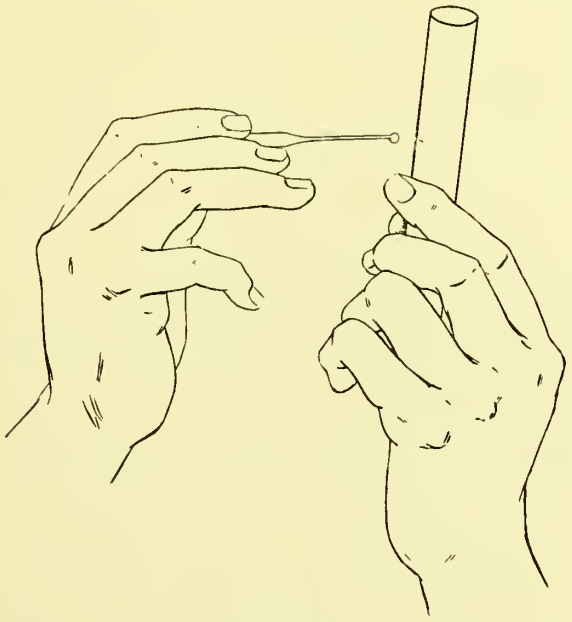


Fig. 14.

to the desired level, and then to direct the fine blow-pipe flame against the tube where air and water meet.

A *third* method, which applies only to thin walled glass-tubing and which involves leaving a rim round the separated ends of the tube, is as follows:—

We take a test-tube—we will suppose that it is a test-tube which is to be cut—bring it to the blow-pipe flame, cut down the gas supply till we have a small pointed flame, and then, holding our test-tube vertically and rotating continuously, allow the small blow-pipe flame to play upon it at the desired level until we have here a thin band of very plastic glass. We now remove the test-tube from the flame, bring our hands together with a sudden movement of compression, and in this way cause the molten glass to fly out all round the tube in the form of a

thin hollow flange. When the glass has cooled down the application of lateral pressure to the tube will snap it off in the hollow of the flange, leaving a rim round each of the separated ends of the tube.

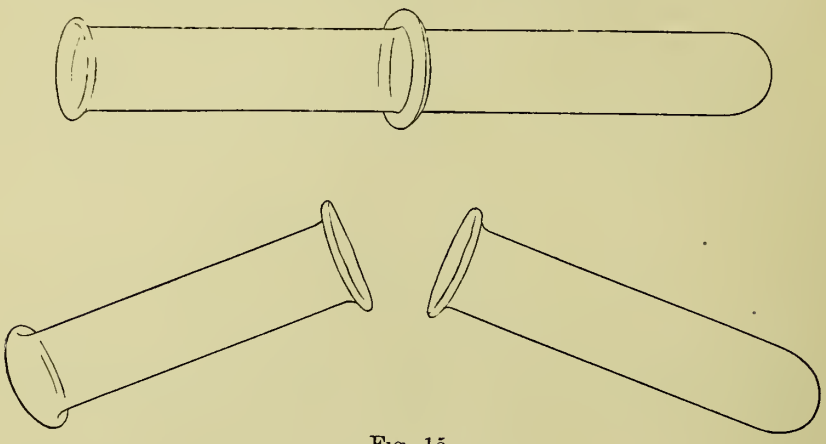


Fig. 15.

5. Method of drawing out glass-tubing.

In drawing out glass-tubing attention must be directed to the following points in addition to the process of annealing already adverted to in *Sub-section* 2, *supra*.

(*a*) In every case one end of the tube must be open. If it is not open pressure will be developed in the tube as the air expands on heating, and as soon as the walls of the tube become plastic the glass will blow out into a bubble and burst.

(*b*) It is convenient in drawing out to deal with only short lengths of tubing.

(*c*) A sufficient length of tubing must be available to prevent the heat from the hot glass spreading to the operator's fingers. If there is not a sufficient length of tubing available on both sides we must provide ourselves with a handle by fusing to the short end any odd piece of glass which may come to hand.

(*d*) The flame must be so regulated as to play upon so much, and only upon so much, of the tube as will suffice to furnish the glass required for the tube which is to be drawn. Where the flame is too large for this purpose it must be cut down, and where it is not large enough to envelop sufficient glass the tube must be held in it obliquely, the positions of the two hands being from time to time reversed so as to bring first one and then the other part of the tube into the hottest of the flame.

(*e*) In order to secure uniform heating of the glass the tube must be made to rotate upon its long axis from the moment of entering to

the moment of leaving the flame. With a view to this the two ends of the tube are to rest upon the middle fingers of either hand while the thumb and forefinger hold the tube in position at either side and impart the rotatory movement. In doing this the fingers of the two hands must move together in accurate time.

It is clear that if the rotatory movement were omitted the tube would be heated only on the one side, with the result that the wall would fall in on that side while the glass on the other would remain quite rigid (Fig. 16, *a*), making it impossible to draw out a tube. It is clear also that if the fingers of the two hands did not keep time in rotating the tube would, as soon as ever the glass became plastic, become twisted and distorted.

(*f*) It is a further requirement—in particular in the case where the tube has been heated in an ordinary Bunsen flame or in the proximal

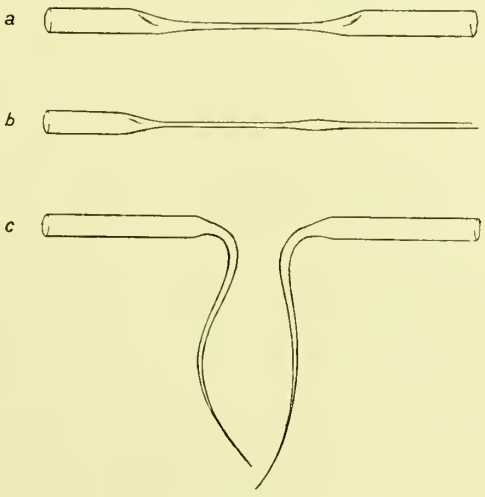


Fig. 16.

portion of a blow-pipe flame—that the tube shall be from time to time displaced laterally so as to bring each portion of the middle segment of the tube in turn into the fringe of the flame—such fringe being of course its hottest portion.

If this precaution is omitted we shall, on drawing out our tube into a capillary stem, obtain in the centre a thicker segment, as in Fig. 16, *b*, and, on either side thinner segments corresponding to the portions of the tube which lay in the hottest portion of the flame.

(*g*) No attempt is to be made to draw out the tube until the glass is quite plastic, and then not until it has been entirely withdrawn from the flame. If an attempt were made to draw out in the flame its heat would, as the tube became narrower and narrower, be concentrated upon a diminishing mass of glass, with the result that this would soon run like water and part in the middle, leaving in each hand of the

operator a tapering end which would sag down by its own weight, furnishing such an aborted and deformed attempt as is shown in Fig. 16, *c*.

(*h*) The rapidity and force with which the tube is drawn out determines the figure of the capillary stem.

Where the force is rapidly applied the capillary stem assumes a tapering figure. This is because in this case the capillary stem is not allowed time to cool and consolidate, and so it yields to the strain and continues to furnish out of its own substance the material for the extension of the tube instead of transmitting the drag to, and withdrawing material from, the mass of molten glass which lies behind.

Where, on the other hand, the pull is only slowly exerted a capillary stem of much more uniform calibre can be obtained. For where each successive length of capillary stem is allowed to cool as it forms, the material for the next following portion will of necessity be provided from the still plastic mass of glass in the neck of the tube.

Where, owing to an error of judgment, the glass has been insufficiently heated and threatens to become rigid before the required length of capillary stem has been drawn out, the situation cannot, as the tyro often hopes, be saved by the exertion of a belated violent pull. The result of such a pull applied when the capillary stem and its widening upper end are already consolidated is to obtain a deformed capillary stem with a bulging portion as shown in Fig. 16, *b*.

(*i*) Where a capillary stem of as nearly as possible uniform diameter is desired, internal air pressure must be brought to bear by blowing into the barrel as the stem is drawn out. The pressure of air thus obtained will, so long as the glass remains plastic, take effect in a differential manner, opening up the narrower portions of the capillary stem until they attain to the calibre of the wider portions.

(*j*) When in drawing out a tube—and this will require attention in particular where we are dealing with a wide and thin-walled tube such as a test tube—we have reason to suspect that the lumen of our tube is no longer accurately circular, and that its walls are no longer everywhere of equal thickness, it will be well in drawing our tube to execute with both hands a to and fro rotatory movement round the long axis of the tube so as to rectify any irregularities of shape and any inequalities in the thickness of the walls.

(*k*) Where a capillary stem with thicker and stronger walls than would be obtained by the simple drawing out of the particular piece of tubing which happens to be at hand is required, the walls of the tube must be thickened before we proceed to pull it out into a capillary stem.

This can be effected by allowing the flame of the blow-pipe to play upon the tube, taking care to rotate this round its long axis without

exerting any pull. The walls will then collapse in such a way as to give us a tube of narrower lumen with proportionately thicker walls. Fig. 17, *a*.

If still further thickening should be required a longitudinal compression may be brought to bear as soon as the glass becomes in the least plastic. An outward bulge, such as is shown in Fig. 17, *b*, will in this way be produced, and when this falls in on further heating in the flame we shall have the very thick walled tube which we desire.

(*l*) Where a capillary stem of reasonable strength and of wider diameter than can conveniently be furnished by the particular piece of glass which is at hand is required, a thick walled bulb must be blown upon the tube, and this must be heated and drawn into the capillary stem.

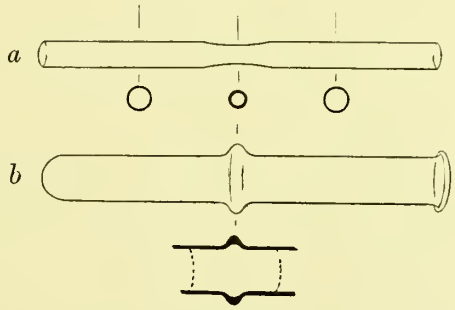


Fig. 17.

The procedure, which is somewhat difficult, and which is only called for when tubing of a larger diameter is not available, is as follows:—

Take a piece of glass-tubing which is not less than 20 cm. in length. Draw out one of the ends into a stout capillary stem and seal up this at the end. This will now serve as one handle. Then rotating the tube and applying the blow-pipe flame to it an inch or more above the point at which it has been drawn out, compress the tube along its longitudinal axis as soon as it shows an indication of becoming plastic. After collecting in this way as much glass as you can into the area which is played upon by the flame bring the whole of this into a thoroughly plastic state, and then, removing the glass from the flame (still keeping up the longitudinal compression), blow it out very gently, making it your aim only to open up the collapsed tube so far as may be required to use up the extra thickness of glass. This done re-introduce the bulb into the flame and work up more glass into it, using the same methods as before, and then blow it out again. When by frequent repetitions of this manœuvre sufficient material has been

collected into the bulb, bring it once more to full red heat, blow into it just sufficiently to correct any irregularities which may have been brought about by the collapse of the walls, and then without delay draw it out into a wide capillary stem, rotating the handle to and fro so as to secure uniformity of bore.

6. Method of bending a piece of glass-tubing.

(*a*) *Procedure where we are dealing with ordinary size of glass-tubing.*—To bend a piece of tubing without producing a kink, *i.e.*, without thinning out the external wall and thickening the inner wall, and generating internal strains in the glass, it is necessary to heat a very considerable length of tubing until the whole has been reduced to a semi-plastic condition. (We can do this best in the large and comparatively cool flame which will be obtained by shutting off the air-blast from the blow-pipe and opening up the gas supply to its fullest.) The lumen of the tube may be kept undistorted if we have previously blocked up one end with a cork, and blow into the tube in the moment of executing the bend. As soon as this has been made, the tube must be re-introduced into the luminous flame in order to efface any strains which may remain in the glass; and finally the tube must be annealed by wrapping it round with paper as soon as it is taken out of the flame.

(*b*) *Procedure when we are dealing with capillary tubes.*—The principles which apply to the bending of wider glass-tubing obviously apply also to the bending of capillary tubes. But we have to take the difference of size into account. We substitute for the luminous flame of the blow-pipe the small, luminous flame of the by-pass of a Bunsen burner; we heat the required length of our capillary tube by passing it backwards and forwards through this flame; and we employ as our bending force the force of gravity. This takes charge as soon as the glass has been rendered plastic by heat, and bends round the tube to a right angle, or to any other angle we may decide upon.

7. Method of drawing out a piece of tubing into a capillary stem, and bending this round so as to form a recurved limb.

Having grasped our piece of tubing with two hands, we introduce it into the blow-pipe flame and draw it out in the middle into a capillary stem of moderate length, and while the glass is still plastic we carry round the barrel-shaped end we hold in our right hand to form such a curve as is shown in Fig. 36, *c*, p. 41, the left hand maintaining for the moment its position. The development of a kink in the concavity of the loop is guarded against, *first*, by postponing the making of the turn until we feel that the glass is on the point of solidifying, and, *secondly*, by taking care, as soon as the loop has been made, to take up its

slack by advancing the left hand in such a way as to make pressure through the capillary stem upon the bight of the loop, pressing it back and opening it up.

8. Method of drawing out a piece of tubing into a capillary stem, and bending this round to form the loop of a spiral.

Taking as before a piece of glass-tubing, and introducing it into the flame, and drawing it out in the middle into a capillary stem of sufficient length to give us a spiral loop of the desired dimensions, we now, while the glass is still thoroughly plastic, proceed as follows: We carry up the left hand, and we carry down the right hand, as indicated by the arrows in Fig. 18, *a*, approximating the two as we do so, so as to make the capillary stem assume the S-shaped contour shown in Fig. 18, *b*. The two ends of the tube are now rotated round their longitudinal axes in opposite directions (as indicated by the arrows), in each case

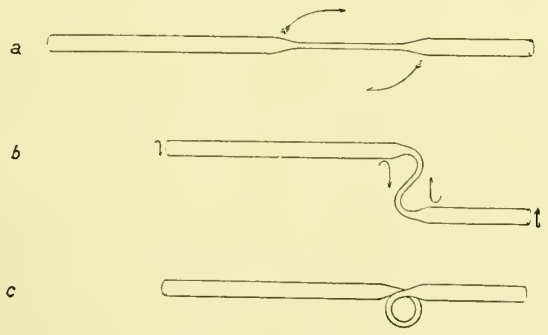


Fig. 18.

through an angle of about 45°. At the same time, by bringing the left hand down and the right hand up, the bights of the two open loops are laid over each other to form a spiral loop whose plane will, when the tube is set up on end, form only a small angle with the vertical, and whose upper and lower segments will be closely applied to the sides of the upper and lower segments of the tube. Actual contact with the sides must, however, be avoided, for this would produce strain and predispose to fracture.

In view of the difficulty which arises in carrying out this technique by reason of the fact that the glass so rapidly loses its plasticity, the beginner will find it convenient to practise making the movements upon material which allows a longer period for thought and reflection between the successive stages of the procedure.

If we take a piece of stout string about 18-20 cms. in length, and encase each end in a rigid stocking of sealing-wax, leaving a length of about 6 cms. free and flexible in the middle, we shall have obtained a material which will be comparable to a piece of glass tubing whose central portion has been softened by heating. The reader will find that he can do his preliminary practice upon this.

9. Method of welding two pieces of glass-tubing.

Where two lengths of glass tubing are to be welded the ends of the tubes must be cut absolutely square, and the far end of one of the two tubes must be rendered air-tight either by sealing it in the flame, or by fitting it to a rubber teat, or by any other method which may happen to be convenient. This done, the straight-cut ends of the two tubes are to be introduced into the blow-pipe flame, and this is directed upon the extreme edges of the tubes until the whole circumference of both has been fused. We now rapidly bring the edges of the tubes together in the flame and direct the blow-pipe flame upon the line of junction until the two pieces of glass are thoroughly incorporated. This done, we remove the tube from the flame and blow into it so as to still further consolidate the joint and to restore the regularity of the outline.

10. Method of sealing off one end of a glass-tube.

(*a*) *Procedure where we are dealing with an ordinary piece of glass-tubing which is to be sealed up at one end and rounded off.*—Where we have to seal up one end of a piece of glass-tubing, rounding off the end after the manner of a test-tube, we begin by drawing out the tube into a capillary stem, as shown in Fig. 19, *a*.

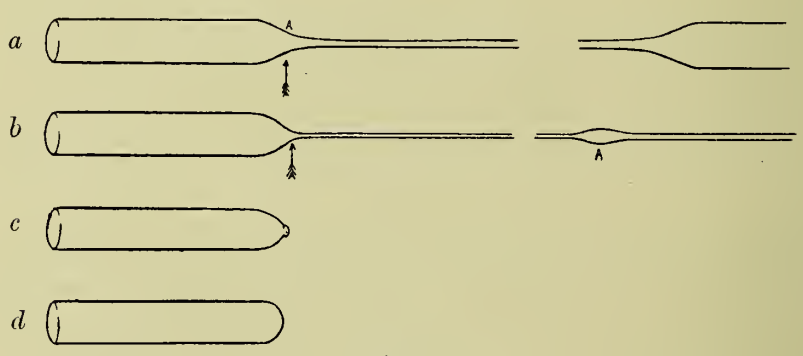


Fig. 19.

Reducing then the size of the blow-pipe flame, we bring this to bear upon the neck of the capillary stem, directing it to the point indicated in the figure by the arrow, and we now draw out into a much finer capillary stem as shown in Fig. 19, *b*.

We continue to remove glass from the bottom of the tube in this fashion until we judge that there is only enough left to round off the end in a satisfactory manner. (Fig. 19, *c*.)

Finally, using the full blast of the bellows and a large flame and allowing this to play upon the bottom of the tube until it has been

brought to a red heat, we blow into the mouth of the tube and so round it off in a symmetrical manner. (Fig. 19, *d*.)

(*b*) *Procedure where we are dealing with a capillary tube.*—Where an ordinary thin-walled capillary tube is to be sealed off at its end, this is done by introducing the very tip of the tube into the edge of a flame and holding it there until the glass fuses and closes the tube.

In the case where the tube is to be sealed off at any other point, the stem is introduced into a flame—preferably a small flame—and it is drawn out by grasping it either inside the flame with the forceps, or just outside the flame with the fingers.

11. Method of sealing up a test-tube or capsule.

The procedure here followed differs from that employed in sealing up the end of an open tube in the respect that we have to take into account the disturbing forces which would be introduced by a positive or negative pressure in the interior of the vessel.

The following points have to be attended to.

If we attempt to seal up a vessel while a positive pressure is being kept up inside, either by the rapid generation of steam or by the leading in of a stream of gas, the walls will not fall together on the application of heat. If the application of heat be persisted in after sealing off is complete and while the glass is still in a plastic condition, the expansion of the contained air will blow out the extremity of the tube into such a thin-walled bubble as is shown in Fig. 21, *e*. If, on the other hand, a negative pressure develops in the interior of the vessel by the cooling of the contained air while the glass is still plastic the walls of the tube will fall in and the glass will be thinned out as shown in Fig. 21, *d*.

Both of these sources of weakness (not to speak of the risk of bacterial contamination which attaches to that sudden inrush of air which would take place on opening a tube in which there was a partial vacuum) can be avoided by postponing the final sealing of every glass vessel until the temperature of the contained air is the same as that of the external air, and then carefully avoiding reheating the contained air. Such reheating may be avoided by employing a very small flame if we have in our preliminary operation reduced the orifice of our vessel to the very smallest dimensions.

(*a*) *Procedure to be followed in sealing up a test-tube.*—The practical application of these principles may be illustrated by the detailed description of the method of sealing up a test-tube. Where this has to be done we grasp the tube above and below and introduce it into the luminous flame of the blow-pipe, rotating it continuously round its long axis. We then gradually turn on the

blast-flame, and, taking care now to keep, in rotating the tube, very accurate pace with both hands, continue the heating until the glass has fallen in sufficiently and has acquired sufficient thickness.

It may now be drawn out into a short stem of moderate thickness (Fig. 20, *a*). As soon as this has become rigid it is reheated and is drawn out again telescope fashion into a capillary stem as shown in Fig. 20, *b*. We now break the capillary stem, set the tube aside to cool down to the temperature of the surrounding air, and then seal off the capillary end in the peep-flame (Fig. 20, *c*).

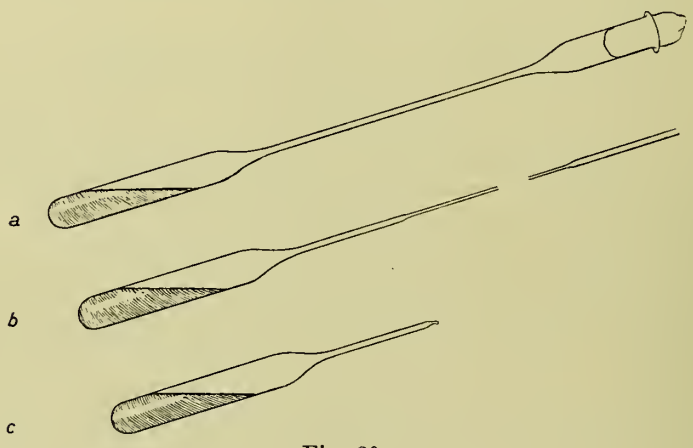


Fig. 20.

In the case where fluid is to be sealed up in a test-tube the following additional points must be attended to:—

Before beginning we must take care to drive off by the gentle application of heat any moisture which may have deposited itself on the walls of the tube.

The test-tube must be held throughout at such an angle to the vertical as will prevent the contained fluid coming into contact with the hot walls.*

The flame must be applied at some distance from the fluid, and heating must not be unduly prolonged for fear of sufficient heat being conducted down to the level of the fluid to crack the tube.

If, as will constantly happen, we find ourselves cramped for space, we must, as a preliminary, fit a handle to the upper end of the tube. In this case we begin by warming the rim of the test-tube very cautiously in the luminous flame of the blow-pipe, and then, gradually turning on the air blast, heat the upper end of the tube till it begins

* Such contact would of course cause the tube to crack, and if sealing were already complete it would lead to explosion.

to soften and fall in. We now apply the piece of glass which we have selected to serve as a handle, in succession to four opposite points on the plastic rim of the test-tube, and draw these points together, leaving between them sufficient vent for the escape of air from the tube.

When the tube has cooled down after sealing, we must in every case put our work to the proof by projecting the contents against the sealed end. If no fluid escapes we may be satisfied that the tube has been properly sealed.

(*b*) *Procedure to be followed in sealing off a capsule.*—Where we have to seal up a capsule we have our choice between sealing it off at the extremity and sealing it at any other point on the stem.

In the former case we shall do well to use the blow-pipe flame. For if we use the cooler flame of a Bunsen burner, or the still cooler

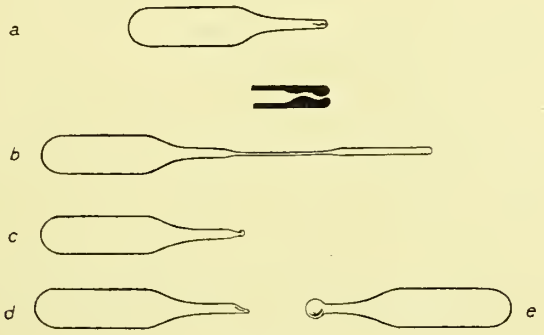


Fig. 21.

flame of its by-pass, there will, when we are persuaded that the tube is quite occluded, often remain open in the thickened end of the stem a very minute channel such as is shown in Fig. 21, *a*; and the thicker the walls of stem the more likely is the accident to occur.

This risk will be entirely avoided if we grasp the end of the stem with the forceps and then draw it out inside the flame into a tube of capillary fineness such as is shown in Fig 21, *b*, and so seal.

12. Method of throttling a capillary tube.

(*a*) *Procedure where we require a sudden restriction of the lumen.*—Where a capillary tube is to be throttled, it suffices to introduce it into the by-pass of a Bunsen burner for a second or a fraction of a second;* to withdraw it from the flame; and then to draw it out with an absolutely instantaneous movement. We then break off the point,

* It is clear that if the capillary tube were held in the peep-flame for a fraction of a second longer than is required for the softening of the glass, its walls would collapse, and we should, on drawing out, obtain, instead of a hollow, a solid hair-fine extremity.

and determine whether the hair-fine extremity we thus obtain (Fig. 22, *B*) is pervious, by blowing down the tube, directing it in the direction of the peep-flame.

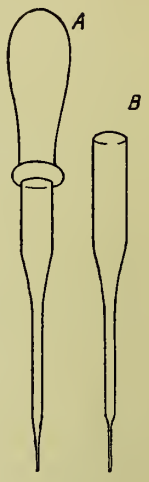


Fig. 22.

(*b*) *Procedure where we require, instead of a sudden, a gradual restriction of the lumen.*—Where a gradual restriction of the lumen, such as is shown in Fig. 22, *A*, is what is required it will be necessary to bring internal air-pressure to bear. Where the capillary stem comes off from a wider barrel, we can effect this by fitting a rubber teat to the barrel, and compressing this at the moment of drawing out the hair-fine filament.

13. Method of drawing out a capillary tube into a rapidly expanding solid point, such as would furnish a satisfactory pricker for obtaining blood from the finger.

Fig. 23.

Where a pricker is required for the purpose of drawing blood from the finger, we heat a comparatively narrow zone of the capillary stem in the peep-flame, and draw it out very rapidly after removing it from the flame (Fig. 23, *B*). We then break off the hair-fine extremity, obtaining in this way a pricker with a sharp and rapidly expanding wedge-shaped point (Fig. 23, *A*), which will enter the skin easily, will open up enough capillaries to furnish a plentiful supply of blood, and will not break off under the skin.

CHAPTER III.

DETAILED INSTRUCTIONS FOR MAKING AND, WHERE REQUIRED, GRADUATING THE GLASS APPARATUS EMPLOYED IN THE METHODS OF BLOOD EXAMINATION HEREAFTER DESCRIBED.

Method of making simple capillary pipettes—Method of making composite pipettes—Method of making throttled pipettes—Method of making looped pipettes—Method of making automatic pipettes—Method of adapting the smaller pattern of automatic pipette for use with fluids which would pass through the throttle—Method of making a many-stemmed pipette—Method of making and graduating a haemocrit pipette—Method of making and graduating a diluting pipette—Method of employing the diluting pipette—Method of making blood-capsules—Method of making a siphon-capsule—Method of making vaccine-bulbs—Method of making conical and rounded-ended tubules.

1. Method of making simple capillary pipettes.

The simple capillary pipette finds manifold applications in connexion with the technique described in subsequent chapters.

Its essential features are (a) the barrel-shaped upper extremity—we may speak of this as the "barrel"—to which a rubber teat can be fitted —and (b) the capillary stem which, when provided with a fiduciary mark, serves for measuring off "unit-volumes" of the fluids we have to deal with.

Simple capillary pipettes are made in pairs. The procedure is as follows:—Glass-tubing of from 5 to 7 mm. external diameter and 3 to 5 mm. bore (in actual practice one selects the particular size of tube which fits most accurately the teat one proposes to use) is cut into lengths of about 9 cm. and a pile of these is placed ready to hand upon the blow-pipe table. Taking these up one at a time, introduce the central portion into the blow-pipe flame, and rotating continuously, heat in each case a band of some 2 cms. width until the glass is so plastic that it can be moved about in any direction. The tube is now raised out of the flame and is then slowly drawn out until the capillary stem is about 350 mm. long. After holding the tube taut for the space of a few seconds to allow of the glass consolidating

in either neck of the tube, we again lower our hands so as to bring the capillary tube into the flame and burn it through in the middle. We thus obtain a pair of capillary tubes.

If we want a capillary pipette with a rapidly tapering stem we draw out rapidly instead of slowly, and if we want the stem of uniform bore we blow down the tube when we are drawing it out. In Fig. 24 a simple capillary pipette is shown *actual size.*

2. Method of making composite pipettes.

Composite pipettes find application where we require to use in combination with a barrel which will fit an ordinary teat a longer and wider capillary stem than would be easily obtained in connexion with a simple capillary pipette.

The barrel and stem are made separately and are then fitted together. (Fig. 25.)

The barrel is made by nicking and breaking off a simple capillary pipette at the point where the neck runs out into the capillary stem.

The stem is made by taking a piece of glass tubing of 10 mm. or more external measurement, or a stout walled test-tube;* drawing it out, after heating a sufficient length of the tube in the blow-pipe flame; and then cutting up the wide capillary tube thus obtained into convenient lengths.

We now fit one of these lengths into the barrel. In order to do this we put a collar of sealing-wax round the upper part of the stem, but not quite at the end; and then pass the other end into the mouth of the barrel, and down the neck until arrested by the collar. We now gently warm the neck of the barrel in the flame of a peep light so as to soften the sealing-wax—and so lute the capillary stem in air-tight, taking care to keep it in proper allineation.

When we employ a composite pipette which has a wide and unrestricted capillary stem we may, with advantage, fit to it a throttled rubber teat such as is described in *Chapter I., Sub-section* 4.

3. Method of making throttled pipettes.

Throttled pipettes find application where we have to conduct a quantitative test which involves the making of a series of successive dilutions of a serum or other fluid, the mixing of these in measured volumes with measured volumes of a reagent, and

Fig. 24.

* Where glass-tubing of large calibre or a test-tube is not available, a capillary stem of the required dimensions can after some practice be obtained by the procedure described in *Chapter II., Sub-section 5l.*

Fig. 25.

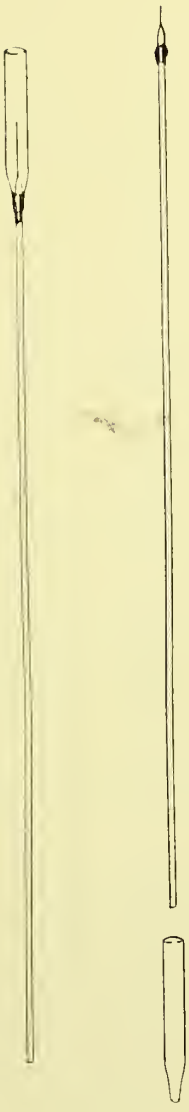


Fig. 26.

Fig. 27.

finally the housing of these mixtures in such a manner as to allow of our reading off the effect produced in each.

The essential feature of the throttled pipette is the throttle which serves as a regulating mechanism and gives accurate control over the movements which we transmit through the rubber teat to air or fluid in the capillary stem of the pipette.

Throttling is resorted to in particular in connexion with wide capillary stems such as are employed in composite pipettes, and we may make three varieties of throttled pipettes, distinguishing them as (*a*) the pipette with the throttle on the proximal end of the capillary stem, (*b*) the pipette with the throttle on the distal end of the capillary stem, and (*c*) the pipette with inserted throttle.

(*a*) *The pipette with the throttle on the proximal end of the capillary stem* (Fig. 26) is a composite pipette, made exactly as described above in *Sub-section* 2 except in the respect that the capillary stem is throttled by the procedure described in *Chapter II., Sub-section* 12*a*, before providing it with the sealing-wax collar and fitting it into a barrel.

While this form of pipette gives very perfect control over the movements of the column of fluid in the capillary stem it compares unfavourably with the two other forms of pipette, in the respect that it is liable to be obstructed if, when operating it, we relax our pressure upon the rubber teat, and so allow water to pass up into the long thin throttle.

The two other forms of throttled pipette are free from this defect.

On the other hand this pattern of throttled pipette has an advantage over the others in the respect that it can be sealed up at both ends of the capillary stem.

Where the throttle is sufficiently long to be reached by the peep-flame of a Bunsen burner through the mouth of the barrel, it can be sealed up by fusing the glass.

Where it is too short it can be sealed by carrying down a little melted sealing-wax upon the end of a capillary tube.

(*b*) *The pipette with the throttle on the distal end of the capillary stem* is throttled by the procedure described in *Chapter II., Sub-section* 12*b*.

(*c*) *The pipette with inserted throttle* (Fig. 27) is made by choosing out a piece of tubing which will comfortably fit into the barrel of the pipette; drawing this out into a capillary stem, and then into a throttle; cutting the tube off short just above the throttle, and finally luting this into the barrel by means of sealing-wax.

4. Method of making looped pipettes.

Looped pipettes find application in connexion with the measurement of the bactericidal power of the blood, and they may also conveniently be

employed wherever we want to draw up into a pipette a sample of a discharge or such like, and to reserve it for examination in an uncontaminated condition.

The essential features of these pipettes are (*a*) *the capillary stem which, when the pipette is employed in connexion with the measurement of the bactericidal power of the blood, serves for measuring and mixing and housing the mixtures of serum and bacterial suspension ;* (*b*) *the chamber which functions, in connexion with the technique just referred to, first as a reservoir for sterile nutrient fluid and afterwards as a cultivation chamber for determining whether the microbes which have been mixed with the serum have or have not been killed by it ;* (*c*) *the glass loop which acts as a trap preventing extraneous air-borne microbes falling into the nutrient fluid ; and* (*d*) *the handle upon which a rubber teat can be fitted.*

Fig. 28.

Looped pipettes are conveniently made in pairs.

Take a piece of tubing about 5 mm. in diameter and about 15 cm. long.

Following the technique described in *Chapter II., Sub-section* 8, make a loop of a spiral on each end of the tube. Then after sterilising the barrel of the tube between the loops by allowing the blow-pipe flame to play upon it, concentrate the flame upon the middle of the tube and draw it out here into a capillary stem some 30 to 40 cm. in length. Then burn the stem across obtaining in this manner a couple of looped pipettes.

5. Method of making automatic pipettes.

The automatic pipette finds application whenever we wish to measure out (*for instance, for the purpose of graduating a pipette or calibrating a tube*) *a standard volume of mercury. By its aid we can also measure out for use a standard volume of a viscid fluid such as lysol.*

The essential feature of the automatic pipette is the fine throttle at the proximal end of the inner tube. In view of the fact that this throttle is made so fine as to be impervious to mercury (*at the pressures we employ*) *while pervious to air, we can by the negative pressure of a collapsed teat get mercury to flow in and fill up the inner tube, the inflow being automatically arrested as soon as the mercury arrives at the throttle.*

The automatic pipette can be made in two different patterns : in a smaller pattern which serves for measuring out, let us say, 5, 10 or

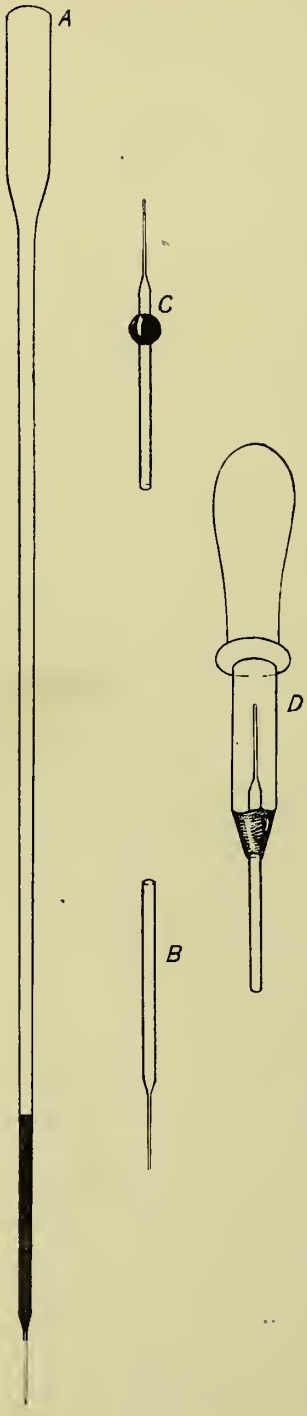


Fig. 29.

25 cmm. of mercury, and a larger pattern which may be conveniently employed with quantities of mercury ranging from 50 to 500 cmm.

(*a*) *Method of making the smaller pattern of automatic pipette.*—Taking a piece of tubing we draw it out into a very thick-walled capillary stem, and throttle it in the manner described in *Chapter II., Sub-section* 12, *a*—drawing it out into an absolutely hair-fine extremity and breaking off the end.

This done, fit a rubber teat to a standard pipette (*vide Chapter I., Sub-section* 6) of, as the case may be, 5, 10 or 25 cmm. capacity; fill it with mercury up to the fiduciary mark; and now transfer the mercury from the standard pipette to the barrel of the capillary pipette you have just made. Having collected the whole pellet into the neck of the capillary pipette, blow into the mouth of the tube in such a way as to force the mercury down to the very extremity of the capillary stem. If it is held back there, as shown in Fig. 29, A, the throttle is fine enough for our purpose.

Keeping the mercury in position against the throttle, now take a glass-cutting knife, and cut across the capillary stem exactly at the proximal end of the column of mercury. There will thus have been obtained a segment of capillary tube (Fig. 29, B), whose capacity will exactly correspond to that of the standard pipette from which we started.

We must now mount our capillary tube. Proceed here exactly as when making a throttled pipette. In other words, place a collar of sealing-wax upon the capillary stem a few millimètres distant from the throttle, and drop the tube, throttle uppermost (Fig. 29, C), into the truncated upper end of a capillary pipette, drawing it

through the neck, and then luting it in position by warming the sealing-wax.

(*b*) *Method of making the larger pattern of automatic pipette.*—Take a piece of glass-tubing and draw it out into a long and roomy and thick-walled capillary stem, and throttle this at its distal extremity. Then fill into the barrel from a standard pipette the desired quantity of mercury. Let it run down the capillary stem for, say, 2 cm., and then arrest it by sealing the tip of the throttle in the flame. Now, holding the tube upright, place a mark (Fig. 30, A) with a glass-writing pencil upon the barrel at the upper level of the mercury.

This done, break off the tip of the throttle, and let the mercury flow out into the stem, stopping its flow as before by sealing up the throttle. Now draw out the tube in the blow-pipe flame, at the point indicated by the fiduciary mark, into a short thick capillary stem (Fig. 30, B), and then draw this out in the peep-flame at any convenient point into a throttle (Fig. 30, C).

This done, allow the glass to cool down completely; then turn up the capillary stem and let the mercury run down until it is arrested by the throttle; cut across the lower stem exactly at the limit of the mercury column; and finally, mount, as in Fig. 30, D, in an enclosing tube.

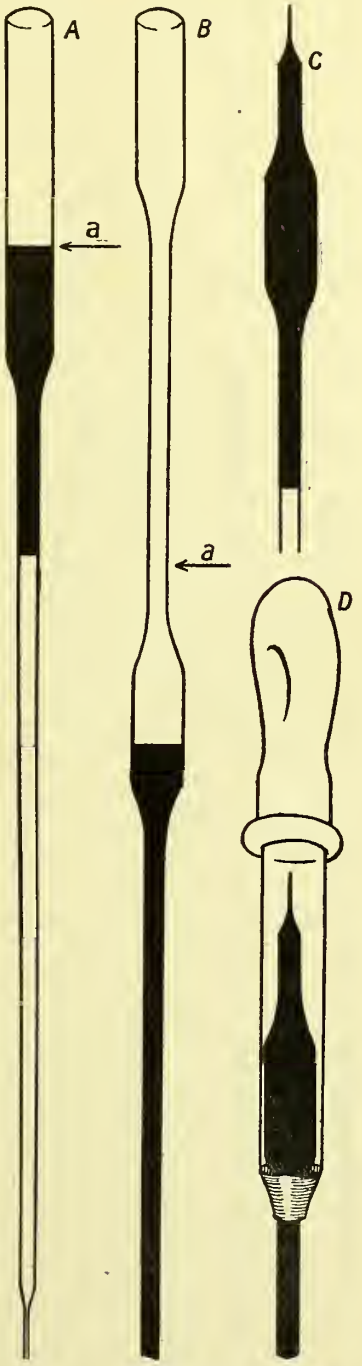


Fig. 30.

6. Methods of adapting the smaller pattern of automatic pipette for use with fluids which would pass through the throttle.

There are two ways of solving the problem which presents itself where

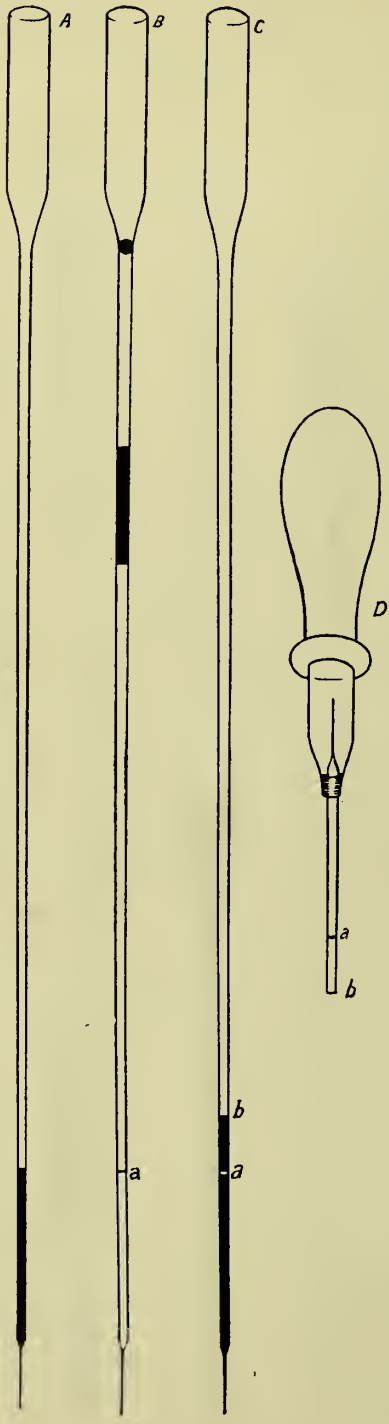


Fig. 31.

we desire to employ the automatic pipette in connexion with watery fluids.

Method No. 1.—We can use a mercury valve. If we proceed by this method we modify the technique described *supra*, *Sub-section* 5, *a*, in the following manner. Instead of cutting across our capillary stem at the upper limit of the mercury immediately after blowing this down into the throttle, we inscribe a mark upon the stem at this point *a*, Fig. 31, B, and then turning the throttle end up we gently shake down the mercury into the barrel of the pipette. Keeping it here we now add to it a small unmeasured globule of mercury (Fig. 31, B), such as we judge will serve the purpose of a valve. We now blow down the combined volume of mercury to the end of the capillary stem (Fig. 31, C), cut this across at the upper limit of the mercury, *b*, Fig. 31, C, and then complete the pipette (Fig. 31, D).

In using the pipette we first prime it up to the division mark *a* with mercury and then fill up with the watery fluid. We may conveniently speak of this as the *mercury-valved automatic pipette.*

Method No. 2.—This method, which was devised by my fellow-worker, Dr. L. Noon, supplies a very convenient way out of the difficulty by sacrificing something of the principle of the automatic pipette. All that is required is, first to break off the tip of the throttle so as just to allow water to pass it with fair ease, and then in employing the pipette to keep a watch on the

column of fluid and arrest it by pressure on the teat as soon as it reaches the constriction at the proximal end of the tube. This is very easy to do, for the inflow checks automatically when the fluid reaches this point.

We may conveniently speak of this pipette as the *semi-automatic pipette.*

Fig. 32.

7. Method of making a many-stemmed pipette.

In this pipette a number of capillary stems come off from a single barrel in such a manner that they can all be filled either together, or in succession, and can afterwards be separately blown out.

The many-stemmed pipette—which is thus a counterpart of the long throttled pipette, which comes into application where it suffices to read off a series of results without blowing out the separate volumes—may with advantage be used in connexion with the measurement of the coagulation-time, and also in connexion with the measurement of the anti-tryptic power of the blood.

We take a fairly stout-walled test-tube. We heat it in a blow-pipe flame, regulating the amount of melted glass in such a manner as to give when we stretch both arms full length a capillary tube measuring about 1 mm. or somewhat less in thickness. We next divide this into lengths of about 9 cm., using a very sharp glass-cutting knife. Or we may break across the tube between finger and thumb, the ends being afterwards trimmed square with the finger nail.

We have now to embed our tubes in plasticine, and by its aid to fit them air-tight into a glass barrel which can afterwards be furnished with a rubber teat.

Taking as much plasticine as would be contained in a cube measuring 1·5 cm. each way, we convert it into a spherical pellet by moulding it between the palms of our hands.

We then make up a set of five tubes; arrange them side by side palisade fashion (as in Fig. 33, A); and holding them in position with the finger and thumb of each hand we bring down our palisade of tubes edge-wise upon the plasticine pellet in such a way as almost to hemisect it (Fig. 33, B). Bringing together, then, the two hemispheres round the tubes (Fig, 33, C), we have them now embedded side by side in a sphere of plasticine.

It will be well to realise what this arrangement gives us. In order to see we have only to take hold of the short ends of the tubes with the thumb and finger of the left hand, and then to make pressure with the thumb and finger of the right hand upon the sphere of plasticine in such a way as to flatten it out into a disc.

As the pressure is applied the tubes will, as the reader will find, open out after the manner of a fan, this being due to the plasticine being driven in like a wedge into the interstices between the individual tubes.

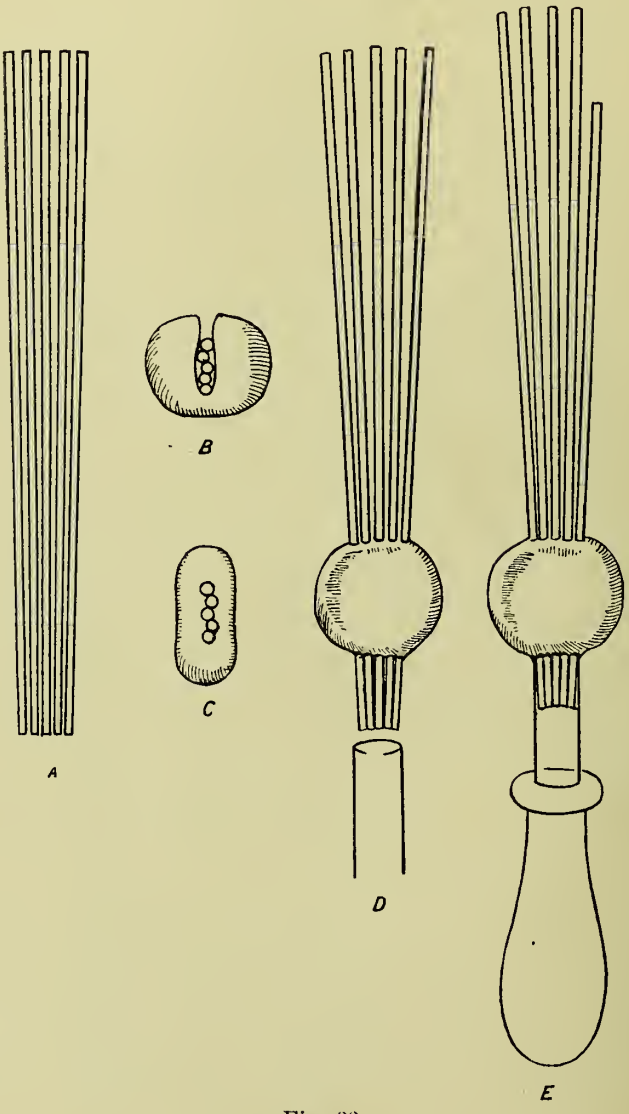


Fig. 33.

Having realised this for future use we restore things to their original condition, and provide ourselves with a glass barrel which can be furnished with a teat and which will hold together the ends of the capillary tubes exactly as our finger and thumb did. A short length

of glass-tubing, whose lumen is just large enough to take in comfortably our five capillary tubes as they lie side by side, is what we require (Fig. 33, D).

Having obtained such a barrel we carry it down over the ends of the capillary tubes and embed it deeply into the plasticine, afterwards carefully moulding this round the tube so as to form an air-tight joint.

We now fit to the barrel a rubber teat, and finally, for a purpose which will appear when the method of employing the many-stemmed pipette comes up for description (*Chapter VII., Section II., Sub-section* 8), we shorten one of our five capillary tubes by trimming off its end. We shall speak of this as the by-pass tube (Fig. 33, E).

8. Method of making and graduating a hæmocrit pipette.

Essential features.—We have here a pipette with a distal throttle upon which are marked off six divisions of equal cubic capacity, and

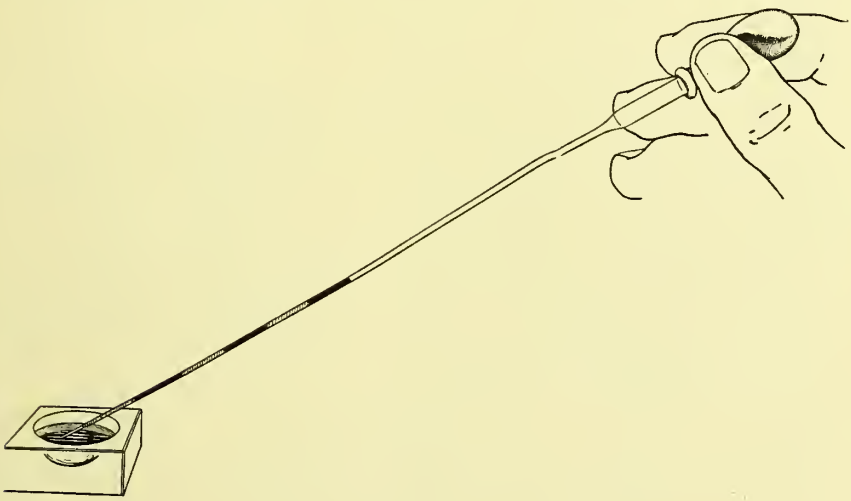


Fig. 34.

another division whose capacity corresponds to the sum of the other six. By the aid of these graduation marks (*a*) *we can measure off a convenient number of unit-volumes of blood,* (*b*) *we can add to these as many unit-volumes of decalcifying solution as may be required to prevent coagulation, and* (*c*) *when the blood corpuscles have settled down, we can read off on the scale the proportion of corpuscles to fluid.*

Take a simple or composite pipette with a fairly wide capillary stem which has been throttled at the distal end by the procedure described in *Chapter II., Sub-section* 12, *b*. Fit to it a rubber teat, and mark off upon the capillary stem a division corresponding to what you judge to be a convenient unit-volume. Now pour a little mercury into a watch glass

or into such a salt cellar as is shown in the figure, and then pour in water. This done, compress the teat with your fingers; introduce the tip of the pipette into the mercury, and let it run up until it is level with your division mark. Then lift the tip of the pipette from the mercury into the water, and let this run up the tube until the distal end of the mercury stands level with the division mark; then push back the point of the pipette again under the cover of the mercury; and follow on thus till you have aspirated into the stem of the pipette three unit-volumes of mercury and three unit-volumes of water.

Removing now the end of the capillary pipette from the water, and keeping the column of fluid accurately in position, mark off upon the stem the upper and lower limits of each volume of mercury.

Finally, relax the pressure of the fingers upon the teat sufficiently to draw the whole column of water and mercury up the capillary stem until its distal end stands level with the sixth division mark. Now complete the graduation by placing a division mark upon the stem opposite the upper level of the column.

9. Method of making and graduating a diluting pipette.*

Essential features.—We have here a pipette by which we can measure off any desired volume of fluid and by the aid of which we can make any dilution in an aseptic manner, arriving at very high dilutions, if we desire them, very unlaboriously.

Take a piece of stout-walled glass-tubing not less than 6 mm. in diameter and not less than 15 cm. long, and draw it out at one end into a thick-walled capillary stem of 15 or more cm. length, and let this taper off a little towards the point.

Draw out the end of this capillary tube into a long throttle and break it off.

Take now an automatic pipette of 25 cmm. capacity, fill it with mercury and pass this down through the open mouth of the tube into the capillary stem. This 25 cmm. of mercury should now, as in Fig. 35, A, occupy almost the whole of the capillary stem.

Make with a blue glass-writing pencil a division mark upon the stem at the upper limit of the mercury. This done, fill up an automatic pipette of 200 cmm. capacity with mercury and let this run down into the tube. Now mark off upon the barrel of your tube the upper limit of the mercury (Fig. 35, B).

This is to serve only as a provisional graduation mark.

Now empty out or draw off into a pipette the mercury from the barrel of the tube, setting it aside carefully until you want it again.

* The reader is recommended to master the general principles of the technique as laid down in *Chapter IV.* before setting out to make or to use a diluting pipette.

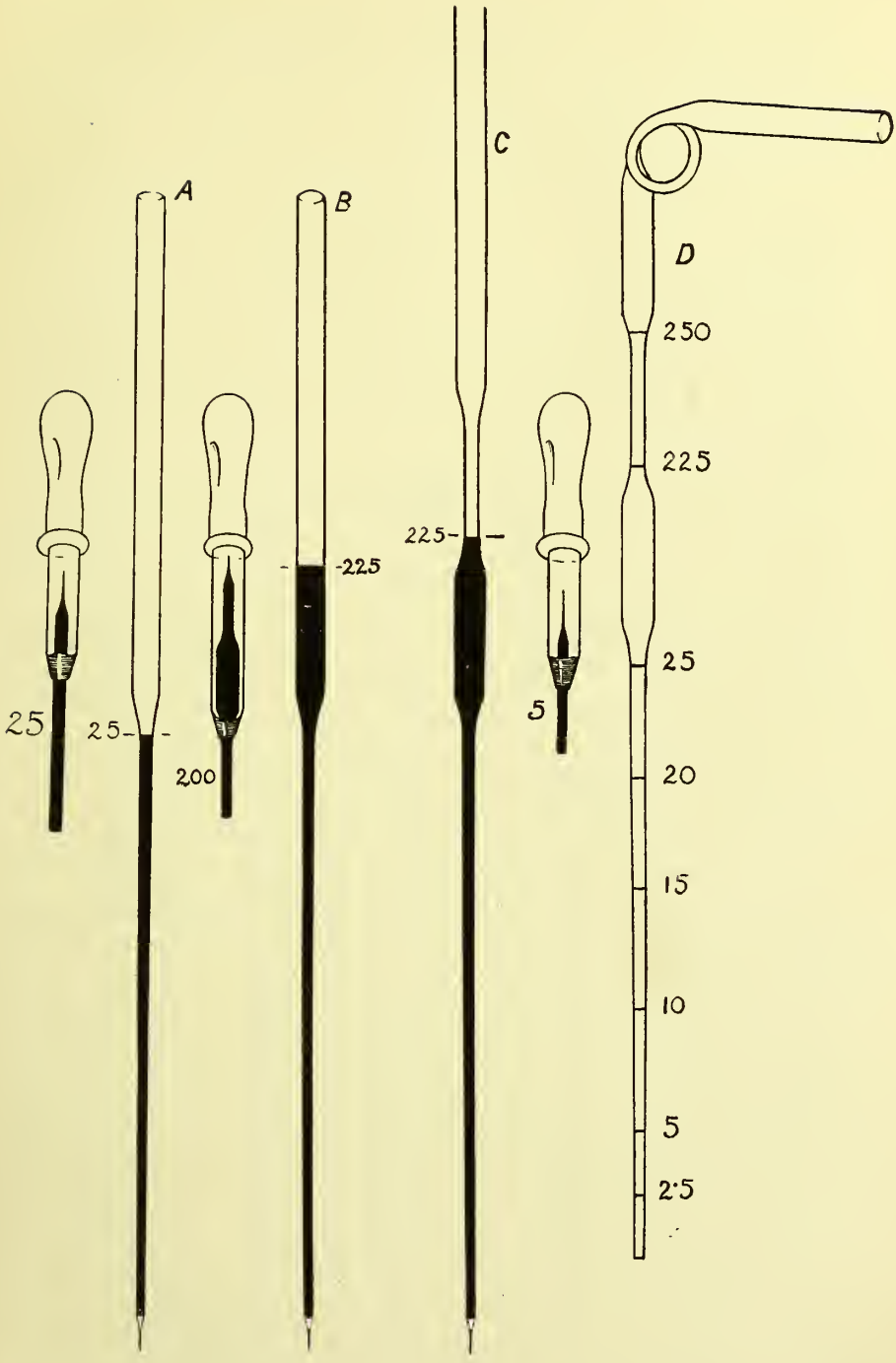


Fig. 35.

Then introduce the diluting pipette which you are fashioning into the blow-pipe flame and heat the barrel immediately above the level of the tentative graduation mark, and draw it out here into such a stout and roomy capillary stem as is shown in the figure (Fig. 35, C). Then, using a simple capillary pipette as a filler, fill in again the mercury which was withdrawn. If the barrel of the pipette has been drawn out at the proper point the upper level of the mercury will now stand somewhere in the lower end of the upper capillary stem.

Now introduce another 25 cmm. of mercury, and supposing that the restricted portion of your tube has the proper capacity, the upper limit of the mercury will fall within it (Fig. 35, D). Mark off that upper limit on the outside of the tube. Mark off also, on the stem of your pipette, the lower limit of the mercury, and then empty out all the mercury.

The next step will be to form a safety chamber, to provide protection for this from bacterial contamination, and to set the handle of the pipette nearly at right angles to the long axis of the pipette. This is to be done by drawing out the upper part of the tube into a stout capillary stem, forming this into a spiral such as is employed in connection with the looped pipette (*Sub-section* 4 *supra*), and then while the spiral is still plastic, carrying round the handle of the pipette through a further angle of 90° to 120°, finally annealing the whole carefully.

The pipette is now practically complete, except for the graduation of the distal capillary stem into five divisions of 5 cmm. and the sub-division of the distal division into two 2·5 cmm. sub-divisions.

The graduation of the stem into 5 cmm. divisions is carried out by introducing 5 cmm. of mercury into the neck of the diluting pipette, running down the mercury to the division mark at the distal end of the stem, and then marking off 5 cmm. divisions on the glass in the manner described in *Chapter IV.*, *Sub-section* 17.

The hemi-section of the distal 5 cmm. division into two 2·5 cmm. divisions is to be carried out in the manner explained in *Chapter IV.*, *Sub-section* 18.

Finally, the capillary stem is to be cut through at the level of the distal division mark; all the graduation marks are indelibly fixed upon the glass by heating them strongly in the flame; and a fairly capacious and tightly fitting rubber teat is to be fitted to the handle.

10. Method of employing the diluting pipette.

The description of the method of employing the diluting pipette ought logically to be postponed until after the exposition of the general principles of the technique of the teat and the capillary tube. It will, however, be more convenient to dispose of this matter here.

Where dilutions have to be made with aseptic precautions, the whole of the pipette—the handle only excepted—ought at the outset to be sterilised by passing it through the flame of a Bunsen burner after careful preliminary washing. When, as will constantly happen, it becomes necessary to re-sterilise the pipette in the middle of operations, it will, when we are dealing with bacteria, which are killed instantaneously by exposure to 100° C., suffice for re-sterilisation to draw up into the pipette boiling sterilised water, and to pass the capillary stem through the flame.

Coming now to the dilution operations. If we require a dilution of 1 in 10 we proceed as follows, varying our technique according as we require a smaller or larger volume of dilution.

In the case where we require a comparatively small volume, we aspirate into the stem of our pipette 5 cmm. of the dilutand, and then, separating off as explained in *Chapter IV., Section* 2, with a bubble of air, we fill in with the dilutand up to the 20 cmm. division mark, and then, separating off by another bubble of air, fill up again to the 25 cmm. division mark.

In the case where we require a larger volume of a 1 in 10 dilution, we fill up with the dilutand to the 25 cmm. division mark, and then, separating off by a bubble of air, fill up with the diluting fluid to the 250 cmm. division mark, taking care to see that the bubble of air which was introduced removes itself out of the way by rising to the surface of the fluid when it enters the bulb of the pipette.

If instead of a 10-fold dilution we want a 100-fold dilution, we follow the same procedure, with the difference that we fill in with the dilutand only up to the 2·5 cmm. division mark.

In the case where we want to make, in an aseptic manner, a geometrically progressive series of dilutions, let us say the series 1 in 10, 1 in 100, 1 in 1,000, 1 in 10,000, 1 in 100,000, and 1 in 1,000,000, we proceed as follows. We provide ourselves with six sterile tubules, and arrange these in order in a tray of plasticine, sloping them to an appropriate angle as in Fig. 65, p. 175. We then fill from our diluting pipette into Tubules 1, 3 and 5, 225 cmm., and into Tubules 2, 4 and 6, 250 cmm. of our sterile diluting fluid, and then withdraw from the three last-mentioned tubes in each case 2·5 cmm. of the fluid. This done we fill into Tubule 1, 25 cmm., and into Tubule 2, 2·5 cmm. of the dilutand. We now, in order to provide for proper mixture of the contents of Tubule 1, draw these up into the diluting pipette, and blow them out again several times in succession; and, after re-sterilising the pipette in boiling sterilised water, which has been kept ready to hand in a test-tube, we do the same with Tubule 2. Following the same procedure, we make from our 1 in 100 dilution, a 1 in 1,000, and a 1 in 10,000

dilution ; and from our 1 in 10,000 a 1 in 100,000, and a 1 in 1,000,000 dilution.

If we do not require the intermediate dilutions we can, of course, arrive at the 1 in 1,000,000 dilution by making only the 1 in 100, and the 1 in 10,000 dilutions.

If further intermediate dilutions, let us say dilutions of 1 in 5, 1 in 12·5, 1 in 25, and 1 in 50 are required, these would be made as follows :—

To make the 1 in 5 dilution, we should fill in the diluting pipette with diluting fluid up to the 225 cmm. division mark, and blow this out into the tubule, stopping when the 25 cmm. division mark was reached. We should then, after emptying the pipette, fill in with the dilutand up to the 25 cmm. division mark, and, after separating off with an air-bubble, fill up again to the same mark, and then blow out into the tubule.

To make the 12·5,- 25- and 50-fold dilutions, we should fill into the tubule in each case 250 cmm. of sterile diluting fluid, and then withdraw from the tubules respectively, 20, 10 or 5 cmm. of fluid, replacing by corresponding quantities of the dilutand.

11. Method of making blood-capsules.

Blood-capsules are used for collecting samples of blood where we want blood fluids for examination. The essential features of the capsule are (*a*) *the upper straight limb which can be drawn out into a pricker,* (*b*) *the recurved limb which makes it possible to fill in the capsule by gravity, without risk of the inflow being arrested by the blood running down and blocking the straight limb which provides an outlet for the air,* (*c*) *the upturning of the recurved limb which prevents the risk of the blood running out when the capsule is laid down on the bench or bed.*

Take a piece of glass-tubing about 0·5 cm. in diameter, whose walls are not less than 0·6 mm. in thickness. Draw it out into a short capillary stem, and break this through in the middle (Fig. 36, *a*). Then re-insert the tube into the flame, and leaving a band of about 2 cm. wide to serve as the barrel of the capsule (such a barrel will have a capacity of about 0·4 cc.), draw out the tube into a capillary stem about 7·5 cm. in length and not less than 1 mm. in diameter (Fig. 36, *b*), and following the technique described in *Chapter II., Sub-section* 7, bend it round so as to form a stout recurved limb lying in the horizontal plane (Fig. 36, *c*) ; and now, before the glass has lost its plasticity, draw the capsule gently upwards so as to set its long axis at an angle of, say, 30° to the horizontal (Fig. 36, *d*). Finally, separate the capsule from the parent tube by burning it through at the point marked by the cross line on the diagram (Fig. 36, *d*).

The separated capsule (Fig. 36, *e*) is completed by drawing out the straight limb into a sharp point as in Fig. 36, *f*, to serve as a pricker (*Chapter II., Sub-section* 13).

Where a number of capsules are to be made in series with the minimum of labour, there is a further point which must be borne in mind. When we are bending round the capillary stem to form the recurved limb of the capsule, the resisting force which we encounter is, of course, derived from the rigid tube which lies in the grasp of our left hand. But in the neck of this tube, where it runs out into the capillary stem, the glass is still in a plastic condition, and hence,

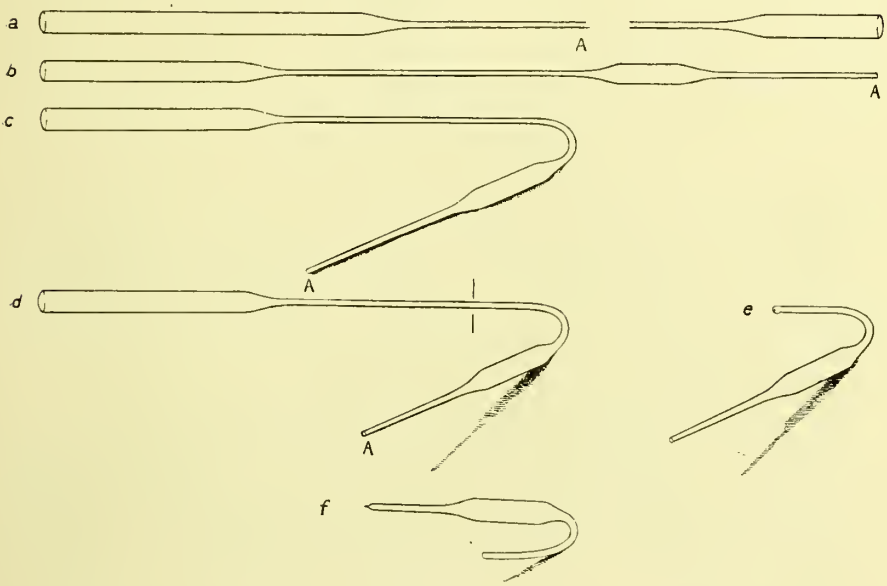


Fig. 36.

if we exert pressure upon it in any other direction than along the long axis of the tube, the capillary stem will be thrown out of alignment, and it will not be possible to use its butt end as the upper limb of our next capsule.

12. Method of making a siphon-capsule.

The siphon-capsule, which was devised by my fellow-worker, Capt. S. R. Douglas, I.M.S., finds application where we have to siphon off and fill into a capsule fluid which is contained in a watch-glass, test-tube or tubule. The siphon-capsule finds further application where we have to fill blood from the finger into a capsule, adding to it, with a view to preventing coagulation, a measured volume of citrate of soda solution. The essential feature of the siphon-capsule is the siphoning arrangement

which has been obtained by bending round the recurved limb in the middle in the form of a knee.

Proceed as in making a blood-capsule, except only in the respect that you here loop back the capillary stem in the form of a simple horse-shoe bend, and that you provide for a longer recurved limb.

Having seen to these points, and having cut free your capsule, take hold of it by the straight limb, and holding the loop disposed

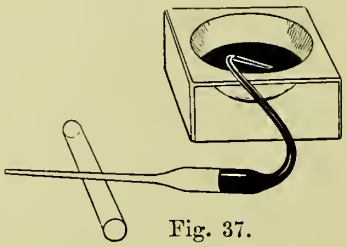


Fig. 37.

horizontally, now pass the central segment of the recurved limb backwards and forwards—following here the instructions given in *Chapter II., Sub-section* 6, *b*—through the by-pass of your Bunsen burner until the distal portion of the tube falls over and sets itself at a right angle to the proximal portion.

13. Method of making vaccine-bulbs.

Take a piece of glass-tubing of appropriate calibre. Draw it out into a thick-walled capillary stem (Fig. 38, *a*), and seal its end (A) in the flame. Then, having marked off with your eye the length of tube which will give you a bulb of the required size, draw the tube out

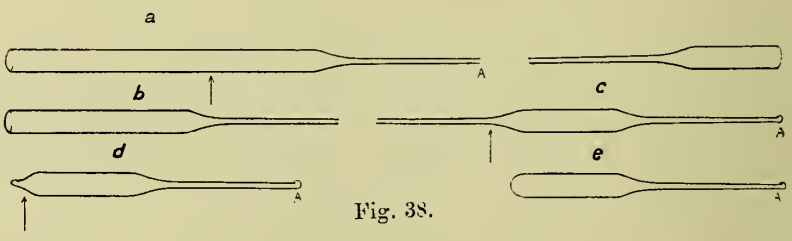


Fig. 38.

again at the point indicated by the arrow in Fig. 38, *a* into a stout capillary stem; and now burn through the glass at the point indicated by the arrow in Fig. 38, *c*, and then allow the flame to play upon the whole butt end of the bulb until the glass has become slightly plastic (Fig. 38, *d*). We now pass the body of the bulb through the flame, and round off its end by means of the internal pressure which is generated by the heating of the contained air (Fig. 38, *e*).

Going back to our original length of glass-tubing, and taking hold of it by the capillary stem (Fig. 38, *b*), which will now serve as the stem of the succeeding bulb, we follow on until we have provided ourselves with all the bulbs which we for the moment require.

14. Method of making conical and rounded-ended tubules.

Tubules find a useful application in connexion with many of the procedures described in the following chapters. Conical-ended tubules serve as receptacles for pooled serum and bacterial suspensions. Rounded-ended tubules may conveniently be employed in conducting the complement deflection test.

(*a*) *Conical-ended tubules* are made out of capillary pipettes by burning these through just below the neck in the flame of a by-pass (Fig. 39, *A*).

(*b*) *Rounded-ended tubules* can be made out of conical-ended tubules by fitting a rubber teat to them, holding their conical ends into the flame of a Bunsen burner, and compressing the teat as soon as the glass has become plastic.

But where a number of such tubules are required, these may, in accordance with a suggestion made by my fellow-worker, Dr. Alexander Fleming, much more conveniently be manufactured by a very simple modification of the method which is employed for making vaccine-bulbs.

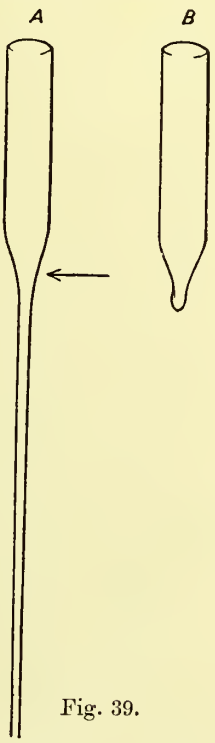


Fig. 39.

Proceeding as described in *Sub-section* 13 *supra*, we make ourselves a somewhat long and narrow bulb, as in Fig. 40, *a*. We then burn

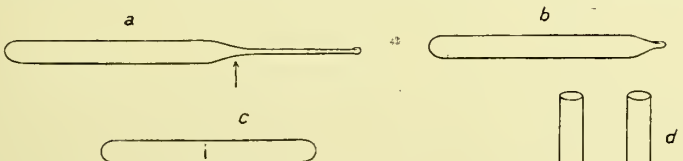


Fig. 40.

through its stem at the point indicated by the arrow and round off the end, shown in Fig. 40, *b*, by the pressure of the contained air. We afterwards apply the blade of the glass-cutting knife to the equator of the double-ended capsule (Fig. 40, *c*) and breaking it across here, obtain a pair of rounded-ended capsules (Fig. 40, *d*).

CHAPTER IV.

GENERAL PRINCIPLES OF THE TECHNIQUE.

Introductory—Method of measuring off and mixing together equal volumes of a test-fluid and a reagent or, as the case may be, equal volumes of a dilutand and a diluting fluid—Difficulties which arise in connexion with ill-fitting teats—Difficulties in connexion with the fiduciary mark—Difficulties in connexion with the control of the movements of the fluid in the capillary stem—Difficulties in connexion with the formation of bubbles—Difficulty which arises in connexion with the re-aspiration of fluid by capillary attraction after a column of fluid has been expelled from the pipette—Difficulty which presents itself when a capillary pipette, which has been charged with a column of fluid and sealed up, is to be fitted with a teat either with a view to (a) *drawing up the fluid into the barrel, or* (b) *with a view to expelling it through the far end of the tube—Difficulty which presents itself when we have to deal with a capillary stem which does not run out into a barrel upon which a teat could be fitted—Difficulty which arises when a workable teat is not to be had—Method of making any dilution which is a simple multiple of* 1*—Method of making a dilution which is not a simple multiple of* 1*—Method of making a series of dilutions which stand to each other in the same relation as the terms in a geometrical progression—Method of intercalating into a series of geometrically progressive dilutions a series of intermediate dilutions—Method of making with the maximum economy of the test-fluid a series of dilutions corresponding to a fraction and simple multiples of that fraction—Method of diluting a more concentrated reagent, so as to make it correspond with one that has been standardised—Method of marking off upon a capillary stem a division corresponding to a standard volume or a series of such divisions—Method of hemisecting a division on a capillary stem into two equal sub-divisions—Method of calibrating a capillary tube.*

1. Introductory.

The features of the technique in its simple form are (*a*) the employment of uncalibrated and ungraduated capillary pipettes for volumetric measurements, (*b*) the use of a rubber teat for drawing up fluid into these, (*c*) the inscription of a pencil line on the stem of the pipette to mark off an arbitrarily selected volume to serve as a unit of measurement, and (*d*) the use of an air-bubble index to divide off from each other unit volumes of fluid as these are drawn up into the pipette, and finally (*e*) certain devices for mixing together the measured volumes.

2. Method of measuring off and mixing together equal volumes of a test-fluid and a reagent or, as the case may be, equal volumes of a dilutand and a diluting fluid.

Take a simple capillary pipette and inscribe with a glass-writing pencil a mark upon its stem at any convenient point—say at about 2·5 cm. from its distal end. Now fit a rubber teat to the barrel of the pipette, and holding it between finger and thumb, in the manner shown in Fig. 34, compress it so as to expel about half the contained air. This done, introduce the mouth of the capillary tube into the fluid which is to be dealt with and, cautiously relaxing the pressure on the teat, let the fluid run up into the tube. As soon as it reaches the fiduciary mark check the inflow. Now, raising the point of the pipette out of the fluid and again cautiously relaxing the pressure, let the fluid run up a little further, say 1·5—2 mm., into the tube, drawing in behind it as it does so a column of air. Having in this way obtained the air-bubble index you require to separate the successive volumes of fluid, introduce the distal end of the pipette into the second fluid—this will of course have been placed ready to hand—and allow it to flow in until, as before, it reaches the pencil mark. Exactly equal volumes of the two fluids will now have been aspirated into the pipette.

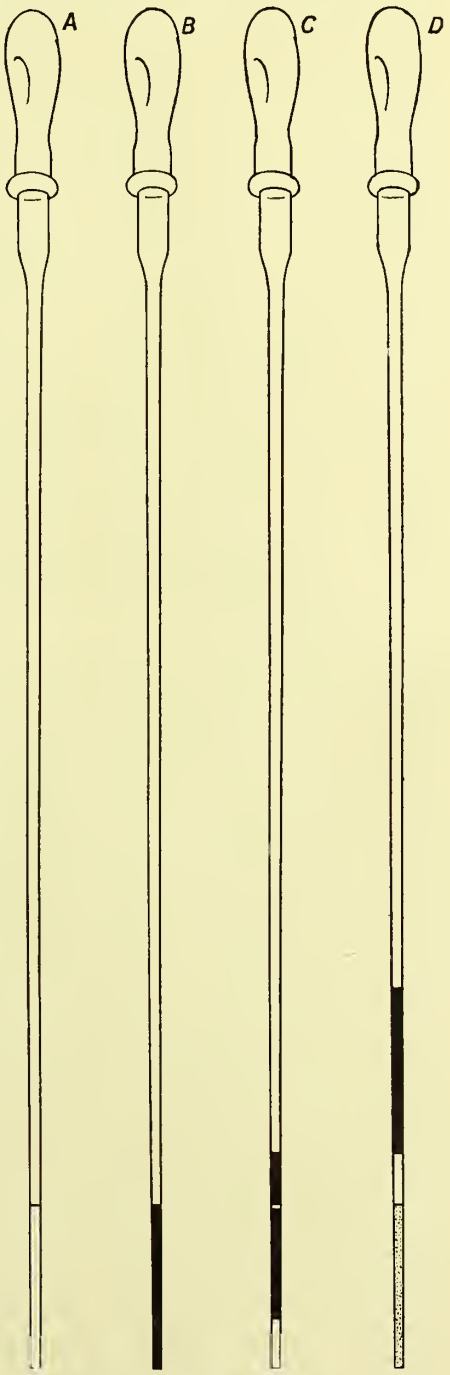


Fig. 41.

These have now to be mixed. This may be done either in the pipette or outside it. If the fluids are to be mixed in the pipette they are drawn up into the neck of the tube. The intervening air bubble will here escape and the fluids can be intimately mixed by alternately relaxing and compressing the teat. If there is no reason for mixing in the interior of the tube the contents are blown out upon a glass slide and mixture is effected by re-aspirating and blowing out again several times in succession.

We have now to deal with certain difficulties which may be encountered in carrying out the technique.

3. Difficulties which arise in connexion with ill-fitting teats.

The most common source of difficulty in connexion with the technique is an ill-fitting rubber teat. The very smallest leak makes easy and accurate working quite impossible. It is therefore advisable before commencing work in every case to test one's teat. This is done by fitting it to a simple capillary pipette, compressing the teat, and then sealing up the orifice of the capillary stem in the flame. If the teat still remains collapsed when the grasp of the fingers is relaxed the fit is everything that can be desired.

Fig. 42.

Where the tube is too small for the teat, and a tighter teat is not available, the leak can be caulked by removing the teat, moulding melted sealing-wax round the rim of the barrel, and then replacing the teat upon the bed of melted wax.

Another way to remedy the leak is to tighten the grip of the teat by fitting over its neck a ring cut off from a piece of rubber tubing or the collar of a discarded teat. (Fig. 42.)

Prevention is however, in this matter, better than cure, and it is well in making pipettes to employ the particular size of tubing which accurately fits the teat one proposes to employ.

4. Difficulties in connexion with the fiduciary mark.

In the ordinary case the unit-volume of fluid which we employ will be that quantity of fluid which will occupy the distal end of the capillary tube as far as the fiduciary mark. We may, however, miscalculate the amount of fluid at our disposal, and may find that it does not suffice to fill up the tube as far as the fiduciary mark. In that case, instead of re-marking the tube, we may substitute for the unit-volume which we had intended to use the volume which is actually available, and arrange matters so as to aspirate into the tube correspondingly reduced volumes of our other fluid. The method employed is as follows:—

After aspirating into our capillary stem whatever volume of fluid No. 1, we happen to have at disposal, we allow it to pass into the tube

as far as the fiduciary mark (Fig. 43, A). As it passes up, it is of course followed by as much air as is required to make up the deficit of fluid.

Keeping the end of the column of fluid steady at the fiduciary mark we now introduce the point of the capillary stem into fluid No. 2 and let this, as it flows in, push the air bubble before it until the proximal end of that bubble comes level with the fiduciary mark (Fig. 43, B). The amount of fluid No. 2 which has been aspirated into the pipette is now exactly equal to the amount of fluid No. 1.

To take in another equal volume we remove the end of the pipette from fluid No. 2 and draw in air until the distal end of the first bubble comes level with the fiduciary mark, and then proceed exactly as in drawing in fluid No. 2.

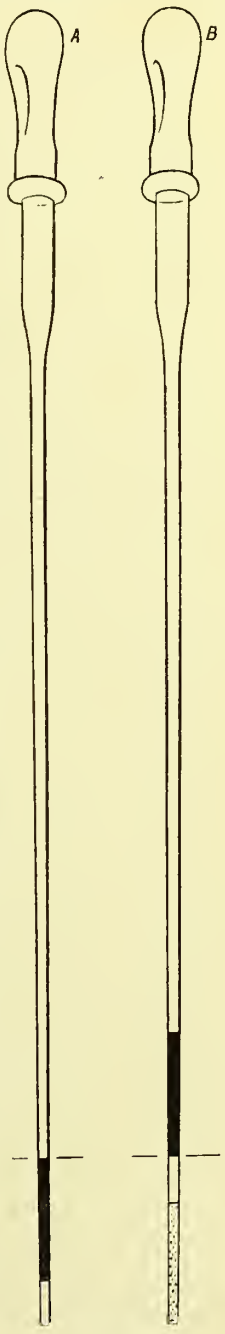


Fig. 43.

5. Difficulties in connexion with the control of the movements of the fluid in the capillary stem.

After a little practice sufficient control over the fingers will have been obtained to regulate the movements of water in the stem of an ordinary capillary pipette. In special cases—in the case, for instance, where we are dealing with a very wide capillary stem, or with a very light fluid like alcohol or ether, or with a very heavy fluid like mercury—there may be a difficulty in properly controlling the movements of fluid in the capillary stem. In such circumstances we may have recourse to any one of the following devices.

(*a*) We may increase the friction in the capillary stem by introducing into it a series of small volumes of fluid separated by bubbles of air. We obtain in this way a very efficient brake, and we may, aspirating these volumes of fluid some little distance up the tube, keep them quite apart from the volumes of the test fluid which follow after.

(*b*) We may use a throttled teat (*Chapter I., Sub-section* 4).

(*c*) We may increase the friction in the tube by throttling the proximal or distal end of the pipette (*Chapter II., Sub-section* 12).

(*d*) Or taking a piece of very fine capillary tube and drawing it out at the end, we may puncture the rubber teat with it and leave it in place so as to provide a by-pass for the air.

6. Difficulties in connexion with the formation of bubbles.

Bubbles may give rise to trouble when we are dealing with blood or suspensions of washed corpuscles, and with albuminous fluids like serum. They may form (*a*) *when we are blowing out our fluids on to the mixing slide*, (*b*) *when we are re-aspirating fluid into the pipette.*

(*a*) In the former case the bubbles will be due to the air being driven out from the pipette when its orifice is still under cover of the expelled fluid.

The theoretically perfect remedy for such bubbling would of course be to relax the pressure on the teat at the very instant that the last of the column of fluid had left the orifice of the pipette.

But inasmuch as it is difficult to make so exact an adjustment, other provision is required. We may arrange matters so that the fluid shall, as it emerges from the tube, flow off rapidly and leave the orifice disengaged.

One method of effecting this is to set the mixing slide up on edge. We can then, having first made contact between the orifice of the pipette and the surface of the slide, blow out the fluid against its sloped or vertical face, letting it flow away immediately after its emergence from the tube.

It is, however, more convenient to allow our slide to lie flat on the bench. In this case, before blowing out the last of our column of fluid, we raise the point of our pipette from the surface of the slide. The fluid will then, on emerging, leave the orifice of the pipette disengaged to seek a lower level and to hang on the under surface of the capillary stem, and we can afterwards touch it off by bringing it in contact with the fluid on the slide (Plate I., Fig. 3, *a*). This, however, will be possible only if the end of the pipette is cut squarely across.

It is clear, that if the end of the pipette were shorn off at a slant and the orifice were directed upwards as in Plate I., Fig. 3, *b*, the emerging fluid would find upon the projecting under lip a point of support, and would then be blown out into a bubble which would burst in a shower of drops. And obviously a similar bubble and shower of drops would be produced also in the case where the orifice is directed downwards, as in Plate I., Fig. 3, *c*.

(*b*) If through want of attention to any of the foregoing points bubbles have been formed upon the slide, the question as to how to avoid re-aspirating them into our pipette immediately presents itself. Two courses are here open to us.

Fig. 1 (a and b) show the condition under which bubbles are formed when respiring fluid into the pipette. (Jupiter 17. Sub-section 6.

Fig. 2 (a) shows the conditions under which the aspiration of bubbles from a drop of fluid into the pipette occurs.

(b) shows how such aspiration may be prevented. (Jupiter 17. Sub-section 6b.

Fig. 3 (a) shows the way in which on the one hand the formation of bubbles on blowing out the pipette, and on the other hand—the respiration of fluid into the tube, may be prevented. (Jupiter 17. Sub-sections 6a and 7.

b) and c) show how when the end of the pipette is shorn off at a slant the fluid does not disengage itself from the orifice, and so gets blown out into bubbles. (Jupiter 17. Sub-section 6c.

PLATE I.

Fig. 1 *a* and *b* show the conditions under which bubbles are formed when reaspirating fluid into the pipette. *Chapter IV., Sub-section* 6*b*.

Fig. 2 (*a*) shows the conditions under which the aspiration of bubbles from a drop of fluid into the pipette occurs.

(*b*) shows how such aspiration may be prevented. *Chapter IV., Sub-section* 6*b*.

Fig. 3 (*a*) shows the way in which—on the one hand—the formation of bubbles on blowing out the pipette, and—on the other hand—the reaspiration of fluid into the tube, may be prevented. *Chapter IV., Sub-sections* 6*a* and 7.

(*b*) and (*c*) show how when the end of the pipette is shorn off at a slant the fluid does *not* disengage itself from the orifice, and so gets blown out into bubbles. *Chapter IV., Sub-section* 6*a*.

Fig 1.

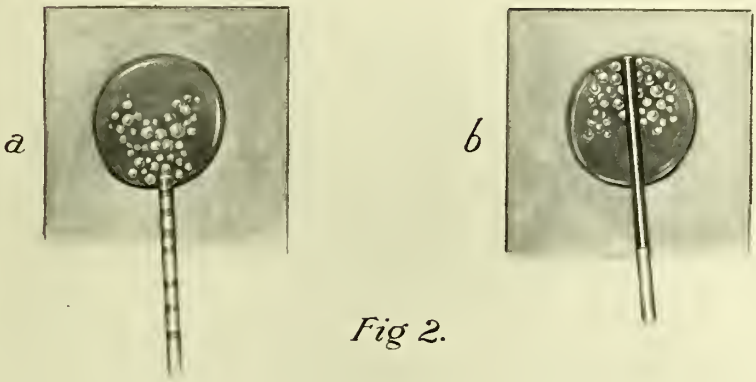
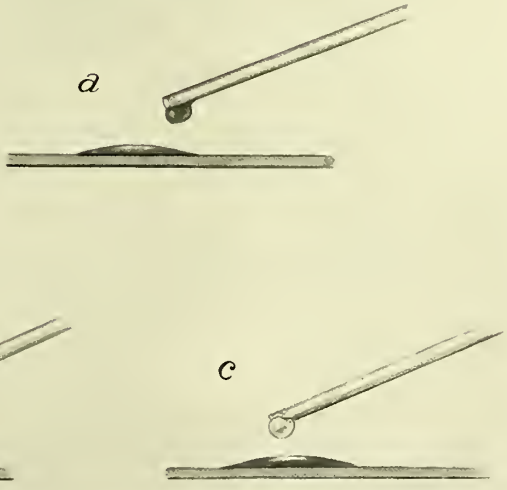


Fig 2.

Fig 3.



[*To face page* 48.

We can tilt up the slide and the fluid will then go to the edge of the drop, the lighter air bubbles remaining at the top.

Or, and this is the simpler way, we may re-aspirate from the far side of the drop. The *rationale* of this last procedure will be understood on referring to Plate I., Fig. 2, *a* and *b*. It will be clear at a glance that when the point of the pipette is so placed there is no room for bubbles to squeeze through between it and the edge of the drop. In fact, we make for ourselves by this device a satisfactory strainer.

Where we have a bubble-free fluid and find when re-aspirating that bubbles are making their appearance in the stem of the pipette, we may be sure that air is entering the tube along with the fluid.

Opportunity for such entrance is afforded when we are filling in from a shallow film of fluid, and the pipette is held too much on the slant. (Plate I., Fig. 1, *b*.)

It is afforded also—and it will be found that this is the most common source of bubbles—when the end of the pipette has been shorn off obliquely, and is so held that part of the orifice is uncovered by fluid. (Plate I., Fig. 1, *a*.)

In either case the remedy will immediately suggest itself.

7. Difficulty which arises in connexion with the re-aspiration of fluid by capillary attraction after a column of fluid has been expelled from the pipette.

This difficulty comes into consideration in every case where we have to be accurate in our measurements, and it presents itself in particular in the case where we have to fill into a pipette a series of unit-volumes of a fluid and to blow these out upon the slide in a series of separate drops. This difficulty is dealt with in exactly the same way as the difficulty which arises from the production of bubbles when expelling the fluid.

To avoid it we raise the point of the pipette from the surface of the slide when expelling the fluid, and then, when the drop is hanging from the under surface of the stem of the pipette, we touch it off by bringing it into contact with the fluid on the slide.

8. Difficulty which presents itself when a capillary pipette, which has been charged with a column of fluid and sealed up, is to be fitted with a teat either with a view to (*a*) drawing up the fluid into the barrel, or (*b*) with a view to expelling it through the far end of the tube.

The real problem which presents itself here is, of course, the problem of displacing the column of fluid in a deliberate as distinguished from a tempestuous manner.

We proceed as follows :—

(*a*) Here we begin by throttling the distal end of the capillary stem (by the procedure described in *Chapter II., Sub-section* 12, *a*); we *then** fit to the barrel a teat in the collapsed condition; we break off the tip of the throttle; and we stand by the by-pass flame of a Bunsen burner, ready to check the in-draught of air by re-sealing the throttle as soon as the column of fluid shall have been drawn up sufficiently far.

Fig. 44.

(*b*) Here we fit to the pipette a perforated teat (*Chapter I., Sub-section* 4), then break off the tip of the capillary stem; then bring the finger over the perforation in the teat and make gentle pressure upon it so as to expel the column of fluid, and then, keeping our finger in place, re-aspirate and re-expel the fluid as often as may be necessary.

9. Difficulty which presents itself when we have to deal with a capillary stem which does not run out into a barrel upon which a teat could be fitted.

When we are confronted by this difficulty we may employ one or other of the following devices.

(*a*) We can block up the mouth of our teat with a piece of glass rod or a closed glass tube or a piece of lead pencil, and then draw out our capillary tube into a hair-fine tube; and finally puncture the rubber teat as shown in Fig. 45, *A*. We can now by manipulating the teat draw up and expel fluid from the capillary stem.

Or, (*b*) we can, as in Fig. 45, *B*, introduce the broken capillary stem into the barrel of a simple capillary pipette and then fit to this a rubber teat; or we can,

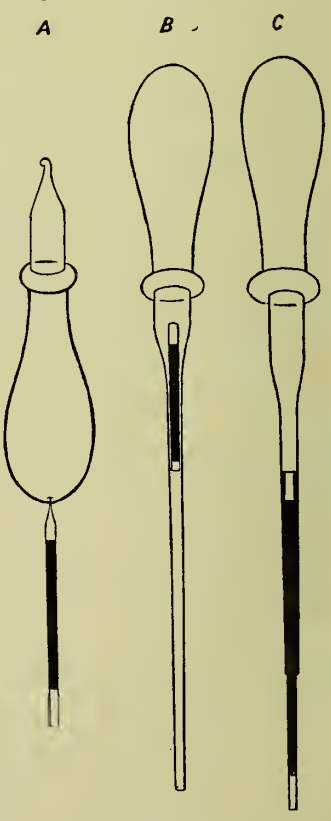


Fig. 45.

* It is clear that if the order of procedure were here reversed we should obtain at the end of our capillary stem not a throttle but an imperforate point.

as in Fig. 45, *C*, pass the broken stem through the neck of a truncated capillary pipette.

10. Difficulty which arises when a workable teat is not to be had.

When a workable teat is not available we may, as a make-shift, use a piece of soft rubber tubing which has been ligatured or otherwise sealed up at one end.

We now pass to the methods of making dilutions in uncalibrated pipettes.

11. Method of making any dilution which is a simple multiple of 1.

The method which has been described in *Sub-section* 2. *supra*, is obviously a method which can be employed not only for making a two-fold dilution but also for making any dilution which is a simple multiple of 1. Where we require a three-fold dilution we take one volume of the dilutand and two volumes of the diluting fluid; where we require a four-fold dilution one volume of the dilutand and three volumes of the diluting fluid, and so on.

Following the same method, we can of course make a dilution of 1 in 10; and again from this by the same method a dilution of 1 in 100; and from this again a dilution of 1 in 1000.

But it will be plain that this will become laborious when we have to arrive at dilutions such as 1 in 1 million, and 1 in 10 million—dilutions which are often required in connexion with bacterial enumerations and which may also occasionally be required in connection with serum-testing. In such a case we may conveniently resort to a *graduated diluting pipette*. For a description of the method of making and manipulating such a pipette, the reader is referred to *Chapter III., Sub-sections* 9 *and* 10.

12. Method of making a dilution which is not a simple multiple of 1.

We express the required dilution in the form of a vulgar fraction, reduce it to its simplest terms, and then, using the same technique as before, take up into the capillary pipette as many volumes of the dilutand as there are units in the numerator of the fraction and add as many volumes of the diluting fluid as suffice to bring up the total number of volumes to the number corresponding to the denominator.

Example 1.—Required, a 4-7ths dilution.—Fill into the pipette first 4 unit-volumes of the dilutand, and add three unit-volumes of the diluting fluid.

Example 2.—Required, a 13-17ths dilution.—Fill into the pipette first 13 unit-volumes of the dilutand and then 4 unit-volumes of the diluting fluid.

Example 3.—You are provided with a 1 in 14 solution, required a 1 in 21 solution.—Fill into the pipette first 2 volumes of the 1 in 14 solution and then 1 volume of the diluting fluid.

13. Method of making a series of dilutions which stand to each other in the same relation as the terms in a geometrical progression.

We here employ either a long throttled pipette of the ordinary type (*Chapter III., Sub-section* 3, *a*), or a semi-automatic pipette (*Chapter III., Sub-section* 6). If we employ the former we take up into the pipette as many unit-volumes of the diluting fluid —separating them off, of course, by bubbles of air—as there are to be dilutions. We then place one or more clean and smooth (*vide Chapter VI., Sub-section* 2) glass slides in front of us and, taking the precautions suggested in *Sub-section* 7, *supra*, blow out this series of unit-volumes as separate drops on to the surface of the glass. (Plate II., Fig. 5, *a*.)

If we employ a semi-automatic pipette we do exactly the same thing except that we draw up and blow out the unit-volumes in a series of separate operations, instead of drawing up, as described above, the entire series of unit-volumes in one operation and blowing these volumes out again in a second.

Having obtained the required series of separate drops of diluting fluid each corresponding to a unit-volume (Plate II., Fig. 5, *a*), we now take up a unit-volume of the dilutand; introduce it into the first drop; mix thoroughly; and then carry on a unit-volume of the mixture into the second drop; mix again there and again carry on a unit-volume; and so on until we reach the end of the series (Plate II., Fig. 5, *b*).

We now have before us a series of dilutions which stand to each as the terms of a geometrical progression in which we multiply by 2. And if we desire to have in each case exactly equal volumes of each dilution all that we shall have to do will be to abstract from the final dilution one unit-volume and discard it.

If a series of dilutions were required which stood to each other as the terms of a geometrical progression in which we multiply by 3, 4 or 5, our technique would be exactly the same except that we should prepare, *in the first case*, a series of drops each corresponding to 2 unit-volumes; *in the second case*, a series of drops each corresponding to 3 unit-volumes; and, *in the last case*, a series of drops corresponding to 4 unit-volumes.

14. Method of intercalating into a series of geometrically progressive dilutions a series of intermediate dilutions.

Where in the case of a series of geometrically progressive dilutions we find the saltus from one dilution to the next too big, it is a very simple matter to introduce intermediate dilutions.

Taking the case where we have a series of 2-, 4-, 8-, 16-, 32-, 64-, and 128-, fold dilutions, we can by starting with a dilution of 1 in 3 and then, using exactly the technique employed for the 2, 4, 8, 16 series, obtain a series of 3-, 6-, 12-, 24-, 48-, and 96-, dilutions to sandwich in between the other series.

15. Method of making with the maximum economy of the test-fluid a series of dilutions corresponding to a fraction and simple multiples of that fraction.

Let us take the case where we have to economise our material and where we desire to make a series of dilutions corresponding to $\frac{5}{6}$, $\frac{4}{6}$, $\frac{3}{6}$, $\frac{2}{6}$, and $\frac{1}{6}$. We should here, following the technique used for making a hæmocrit pipette (*Chapter III., Sub-section* 8), divide the stem of a pipette into six equal divisions. We should then fill into the pipette test fluid up to the fifth division and complete to the sixth division with diluting fluid. This would give us a mixed $\frac{5}{6}$ dilution. Blowing it out on a slide and mixing it we should then draw up four volumes of the mixture, complete with diluting fluid up to the fifth division. This would give us a $\frac{4}{6}$ dilution. After blowing this out on an unused portion of the slide, we should now take up three divisions and complete to four volumes, obtaining a $\frac{3}{6}$ dilution. By taking up two volumes and completing to three we should arrive at a $\frac{2}{6}$ dilution. And finally, by taking up one volume of the last mentioned dilution, completing to two divisions, we should arrive at a $\frac{1}{6}$ dilution.

We should in this manner have before us in each case a series of drops of fluid corresponding to the required series of dilutions, each consisting of two unit-volumes. Completing this with two unit-volumes of the test fluid, we should have a series corresponding to terms of the arithmetical progression $\frac{6}{6}$, $\frac{5}{6}$, $\frac{4}{6}$, $\frac{3}{6}$, $\frac{2}{6}$, and $\frac{1}{6}$.

16. Method of diluting a more concentrated reagent, so as to make it correspond with one that has been standardised.

The technique which is here in question, the principle of which was suggested to me by my late fellow-worker, Dr. Corbyn, may perhaps be best elucidated by considering the procedure in the case where we have a normal solution of oxalic acid, and a hyper-normal solution of sulphuric acid, and where we are required to dilute the latter so as to correspond in strength with the former.

The ordinary procedure would here be to make up a somewhat hyper-normal solution of caustic soda or potash, to titrate this against normal oxalic acid, to standardise in the light of this titration the strength of the alkaline solution by appropriate dilution; then to titrate

the hyper-normal sulphuric acid against the standardised alkaline solution, and finally, in the light of this titration, to standardise the sulphuric acid by appropriate dilution.

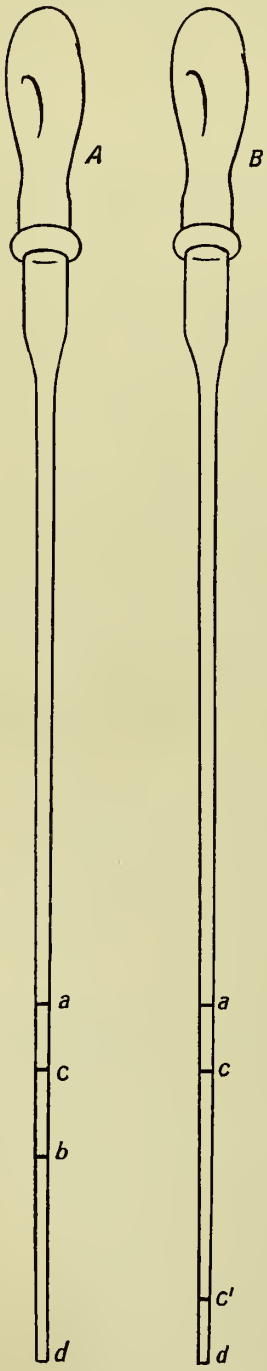


Fig. 46.

Where comparatively small quantities of standardised acid are required the following procedure may be substituted for that just indicated.

Taking a series of watch glasses and a simple capillary pipette upon which we have placed a mark (*a*) corresponding to any convenient unit-volume, we aspirate into the pipette a certain number of volumes of the standardised oxalic acid and blow these on to a slide in the form of separate drops.

Having washed out the pipette we now fill it up to *a* with a hypernormal alkaline solution, mix it with one of the unit-volumes of acid, and then test with litmus paper.

Having satisfied ourselves that this mixture has an alkaline re-action, we place a second trial mark (*b*) upon the capillary stem, somewhat distal to the first.

Drawing up now our alkaline solution to this mark, we blow it out and mix it with one of our unit-volumes of oxalic acid and test the mixture with litmus.

Proceeding in this manner, by trial and error we finally hit off the proper position of which the fiduciary mark (*b*) ought to occupy to indicate to us the volume of alkali which just neutralises the unit-volume of acid. Having found this point, we efface all the division marks except *a* and *b*, and then pass to the next series of operations.

Using now the fiduciary mark *b* as our delimiting mark, we measure off a series of unit volumes of the alkaline solution and then (employing, if such is available, a different coloured pencil for our new set of fiduciary marks) proceed to find by trial and error the volume of our sulphuric acid which will exactly neutralise the unit-volume of the alkali. In

conformity with the fact that we are working with a hyper-normal solution of sulphuric acid this third division mark *c* will, of course, fall distally to *a*, but not necessarily distal to *b*. When its position has been found our preliminary operations will have been completed, and it will be clear that the volume of our hyper-normal sulphuric acid solution which will fill the stem of pipette from its orifice *d* to *c*, must contain an amount of acid which is the equivalent of the volume of normal acid which would fill the tube from *d* to *a*.

We can now in any titrating proceeding employ our hyper-normal sulphuric acid or any aliquot dilution of this, provided that in balancing this acid against alkali we take of the acid always the volume *d* to *c*, and of the alkali the volume *d* to *a*.

Or, if we prefer to do so, we may make up a small stock of normal acid by taking an aliquot number of unit-volumes (*d* to *c*) of acid, and adding to these afterwards for each unit-volume of acid taken the complement (*c* to *a*) of distilled water which is required to convert it into a normal acid.

We obtain the desired number of unit-volumes (*d* to *c*) of our hyper-normal sulphuric by aspirating this number of volumes into the pipette, dividing off the several volumes by bubbles of air. We then blow out the combined volumes into a watch glass.

The required number of volumes of diluting fluid can be obtained by the self-same procedure by marking off on the end of the stem a length *d* to *c'*, equivalent to *c* to *a*. This is very simply done by drawing in a column of fluid *d* to *c* and then carrying along the proximal end of the column till it comes level with *a*. When this has been done it is clear that the distal end of the column of fluid will be our guide to the point *c'*.

17. Method of marking off upon a capillary stem a division corresponding to a standard volume or a series of such divisions.

Take a capillary pipette and throttle its extremity. To the barrel of this pipette now transfer from an automatic measuring pipette the standard volume of mercury which is required. Now cause this to enter the stem of the pipette and allow it to run down to any convenient point. Holding the tube horizontal, the upper and lower limits of the mercury column are now to be marked with a glass-writing pencil. The space between these will obviously correspond to the required volume. Further divisions if required are readily obtained by moving the mercury along the tube.

18. Method of hemisecting a division on a capillary stem into two equal sub-divisions.

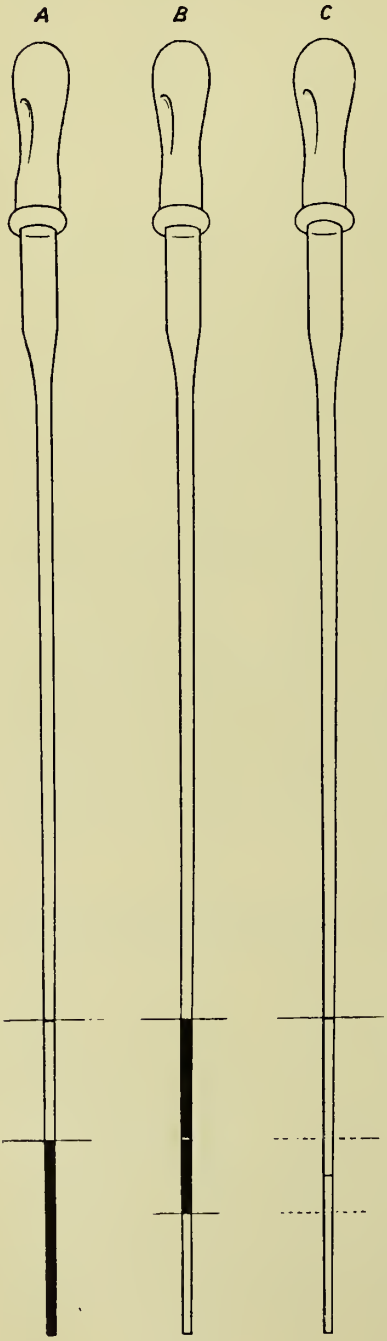


Fig. 47.

For the purpose of describing the technique we may here conveniently consider the case where a capillary stem has been divided into 5 cmm. divisions, and we desire to divide the terminal division into two sub-divisions, each having a capacity of 2·5 cmm.

We begin by drawing up into the stem of the pipette a quantity of mercury corresponding as near as we can guess to 2·5 cmm. The point corresponding to the proximal end of the mercury column may now be tentatively indicated upon the outside of the capillary stem by a light pencil mark. This done, the column of mercury is displaced until its proximal end stands level with the first 5 c.c. division mark. If the distal end of the mercury column now coincides with the fiduciary mark, this will be the desired 2·5 cmm. sub-division mark. If it does not coincide, the desired point will be situated to all intents and purposes halfway between the point now indicated by the distal end of the mercury column and the point indicated by the above-mentioned fiduciary mark.

All these operations will be facilitated if our capillary stem extends some little distance beyond the division which we are hemisecting; for we can then throttle the end of our tube, and displace our mercury column by gravity instead of by means of a teat.

19. Method of calibrating a capillary tube.

The bore of a capillary tube can be measured in a variety of different ways.

(*a*) Where the thickness of the wall is negligible we may make our measurement by the aid of such a gauge as is employed for the measurement of wire. Failing a wire gauge we may lay down our capillary tube upon a finely-divided rule, or where we are dealing with a number of tubes of sensibly the same size, we may range them side by side upon an ordinary carpenter's rule, and so arrive at the average diameter.

(*b*) In the case where the thickness of the wall is not negligible we may measure the bore by passing down into it wires of known thickness, finding out in this way what is the thickest wire which will enter the broadest part of the tube, and what is the thickest wire which will traverse the whole length of the tube.

Or, (*c*) as an alternative to making such a maximum and minimum determination, we may introduce into the tube a standard volume of mercury from an automatic pipette, and then arrive at the average diameter of the tube by measuring the length of the mercury column—calculating in accordance with the formula:—

$$\text{Diameter} = \sqrt{\frac{\text{Capacity in cubic millimetres}}{\text{length of column} \times 0{\cdot}78}}$$

Example—The quantity of mercury introduced is 5 cmm., and the length of the mercury column is 58 mm. What is the average diameter of the portion of the tube which is occupied by the mercury?

$$\text{Diameter} = \sqrt{\frac{5}{58 \times 0{\cdot}78}} = \sqrt{\frac{5}{45}} = \sqrt{\frac{1}{9}} = \frac{1}{3}$$

Answer—0·3 mm.

CHAPTER V.

METHOD OF OBTAINING BLOOD FOR EXAMINATION, OF FILLING IT INTO A BLOOD-CAPSULE OF PREPARING A "POOLED SERUM" AND OF JUDGING OF THE CHARACTERS OF THE BLOOD BY NAKED EYE OBSERVATION.

Method of obtaining blood from the finger—Method of filling the blood into a capsule, sealing it up, and drawing off the serum—Method of preparing a "pooled serum"—Method of affixing a label to a blood-capsule and of assembling a number of blood-capsules upon a labelling card—Method of drawing blood off in an aseptic manner from the finger—Method of obtaining uncoagulated blood—Inferences which can be drawn from naked-eye inspection of the character and behaviour of the blood as it flows from the finger or lies in the capsule—Aspect of the blood in the capsule after coagulation is complete—Aspect of the blood in the capsule where agglutination of red blood corpuscles takes place under the influence of severe microbial infection—Aspect of the blood in the case where blood coagulation has been suspended by an addition of a decalcifying reagent.

1. Method of obtaining blood from the finger.

Blood in quantities, such as are required for any one of the examinations which are described below, can be readily obtained by pricking the finger with a glass pricker such as is made by drawing out a piece of capillary tubing (*Chapter II., Sub-section* 13). Where blood is to be filled into a blood-capsule, the pricker, into which the straight end of the capsule runs out, may conveniently be used (*Chapter III., Sub-section* 11).

The prick is best made on the lateral aspect of the finger, in the neighbourhood of the nail. It may ordinarily be made on the upper (radial) side of the finger; but it will sometimes, for instance in collecting blood for the preparation of washed corpuscles, be more convenient to prick into the ulnar side of the finger, so that the blood may drop off as it flows out.

Preliminary to pricking the finger, or if this is preferred, immediately afterwards, a narrow bandage is to be wound tightly round the finger in such a way as to confine the blood in the distal phalanx. When the finger has been sufficiently deeply punctured and the blood has been

effectively confined by the ligature, gentle pressure either directly applied to the pulp of the finger, or indirectly applied by bending in the distal phalanx upon the next will, except in the case where the patient is ex-sanguine, furnish ample blood to fill into a capsule. If more should be required, it can be obtained by relaxing the pressure of the bandage and reapplying it after blood has re-entered the finger.

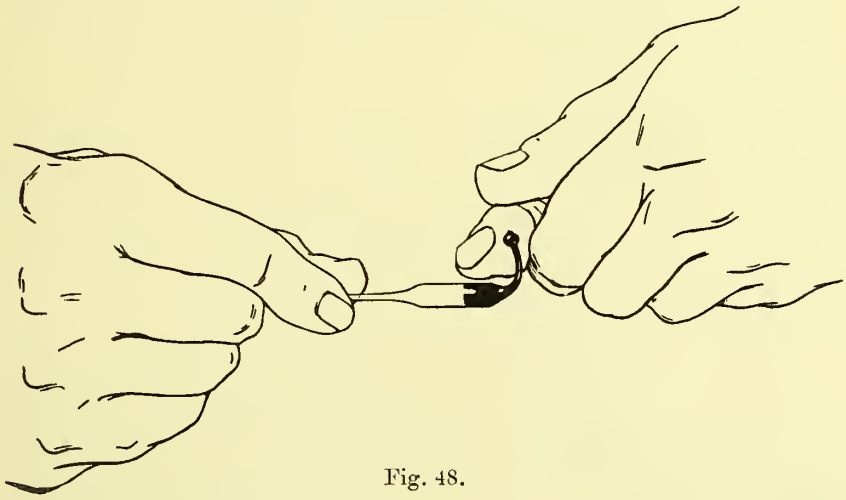


Fig. 48.

2. Method of filling the blood into a capsule, sealing it up, and drawing off the serum.

Break off both ends of the blood capsule, paying special attention to breaking off the pricker in such a way as to provide a free vent for the air through the straight limb of the capsule.

Holding now the blood capsule horizontal and below the level of the finger (Fig. 48), apply the tip of the recurved limb to the edge of the drop of blood. This will now run in by capillary attraction and siphonage, the air escaping through the straight limb of the capsule.

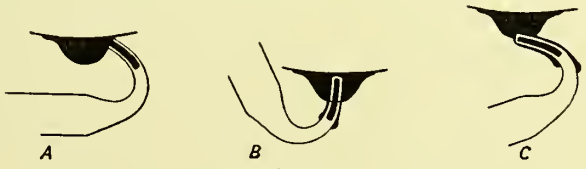


Fig. 49.

In the case where we have pricked the lower aspect of the finger and the drop of blood is hanging down from it, the following must be attended to :—

If more than the tip of the recurved limb is brought into contact with the hanging drop, as in Fig. 49 C, or if the tip is plunged into the

depth of the drop as in Fig. 49 B, the blood will run to waste over the outside of the tube. If on the other hand the tip of the recurved limb is applied to the base of the drop, as in Fig. 49 A, no blood will be lost.

If we should have to remove and reapply the bandage for the purpose of obtaining a further supply of blood, we may lay down the capsule on the bench or bed without fear of the blood running out. This will be effectually prevented by the upturning of recurved limb. Enough blood will have been filled into the capsule when it is half or two-thirds full.

For sealing up the capsule we employ a small steady flame, either the by-pass of a Bunsen burner, or where this is not available a night light or a candle whose wick has been cut off short, or a wax match held upright. We begin by warming the capsule at the point where it runs out into the straight tube, and after thus rarefying the air, we draw out in the flame at a point a little distal to this.

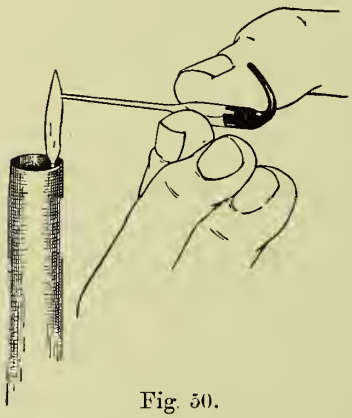


Fig. 50.

The greatest care must be taken to avoid heating the blood. It is for this reason that we have to avoid using a large unsteady flame such as that of an ordinary spirit lamp; and it will be well, when sealing, always to interpose the fingers between the flame and the blood (Fig. 50). After sealing off the straight limb we wait a moment to allow the imprisoned rarefied air to cool. As it cools and contracts it sucks back the blood into the body of the capsule and leaves the orifice of the recurved limb free.

When the capsule is to be sent by post the orifice of the recurved limb is to be sealed up by fusing it in the flame; or it may be closed with sealing-wax, or, where a candle has been used, it may, after extinguishing the candle, be dipped into the pool of liquid paraffin round the wick.

The capsule is now set aside to allow the blood to coagulate and the serum to separate out. Coagulation can be hastened by placing the blood capsule in the incubator, and the separation of serum from the clot can be hurried on by centrifugalisation, the capsule being suspended in the bucket of the centrifuge by its recurved limb.

When the serum has separated out from the clot it may be drawn off by making a nick upon the capsule at the point where it runs out into the straight limb, breaking it off there, and then inserting the end

of a capillary pipette between the clot and the wall of the capsule. There will be plenty of room for the capillary pipette to pass if the capsule has not been filled too full.

3. Method of preparing a "pooled serum."

Where in connexion with a blood examination we employ a control, and a single control, we may best obtain that single control by pooling a number of normal sera. We provide ourselves for this purpose with a number of normal bloods, and when clotting has taken place we draw off from each with a capillary pipette, which we have provided with a fiduciary mark, one or more unit-volumes of serum—taking, of course, the same number of volumes from each—and we mix the sera in a conical tubule (*Chapter III.*, 14 *a*).

4. Method of affixing a label to a blood-capsule and of assembling a number of blood-capsules upon a labelling card.

The simplest way of labelling a blood-capsule is to inscribe the patient's name upon it with a blue glass-writing pencil. Where a glass-writing pencil is not at hand the name may be written on a slip of paper; this slip may be transfixed by the recurved end of the capsule as shown in Fig. 51. The necessary holes can be pricked into the paper with the straight end of the capsule (and in this case it will be well before threading the capsule into the holes to turn the slip of paper round so that the funnel-shaped openings may be directed towards us). Or again, the boring of the holes and the threading in of the recurved end of the capsule may be done in a single operation, if after sealing the end of the recurved limb in the flame we instantaneously apply the hot end to the slip of paper and let it burn its way through from front to back and from back to front in a single sweep.

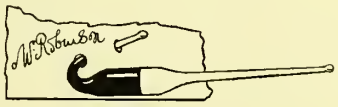


Fig. 51.

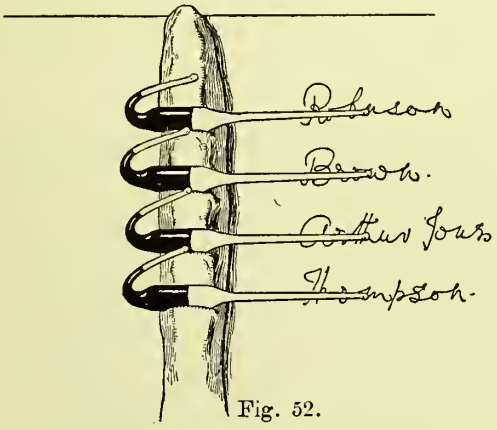


Fig. 52.

Where—as for instance in an out-patient department or in going round the wards—a number of bloods are being collected at one and the same time, it is convenient to assemble the bloods on a roll of plasticine affixed to such a labelling card as is shown in Fig. 52.

Where this method is employed the straight limb of the capsule may conveniently be plugged by pricking it into the plasticine roll, and the retraction of the blood from the orifice of the recurved limb will be provided for if the air in the capsule has been warmed by the heat of the fingers.

5. Method of drawing blood off in an aseptic manner from the finger.

Where, as for instance for the purpose of the measurement of the bactericidal power of the blood, or for the sake of preserving the blood unaltered for the longest possible period, it is desirable to draw off blood in an aseptic manner, this can be done by sterilising the finger (either by flaming it with alcohol, or passing it repeatedly through the flame of a Bunsen burner) and making the prick into the under surface of the finger, employing of course for the reception of the blood a sterile capsule which has been opened up with aseptic precautions.

6. Method of obtaining uncoagulated blood.

Having provided yourself with a 5% solution of citrate of soda, and a simple capillary pipette which you have furnished with a rubber teat and a fiduciary mark, puncture the finger with a pricker, and then draw up into the pipette first 1 unit-volume of the citrate of soda solution and then 4 volumes of blood, mixing together thoroughly by blowing out upon a slide and re-aspirating several times in succession. The uncoagulated blood which has thus been obtained may now be sealed up in the pipette or in a blood-capsule. It may then, after the corpuscles have settled to the bottom, usefully serve for a naked eye estimation of the proportion of white to red corpuscles (leucæmia—*vide* Plate II., Fig. 6), or for the preparation of satisfactory blood-films (severe anæmia—*vide infra, Chapter VI., Sub-section* 8).

7. Inferences which can be drawn from naked-eye inspection of the character and behaviour of the blood as it flows from the finger or lies in the capsule.

When blood wells up freely under slight pressure from a trifling puncture; when, in spite of the careful removal of all moisture from the skin, the blood, instead of shaping itself into a globule, spreads out in a thin layer over the surface; or when, though it first shapes itself into a globule, the blood breaks away under the smallest impulse and streams down the finger; and, lastly, when, after drawing blood and allowing a certain interval to elapse, we find on re-application of the pressure that the blood gushes out as before, it may be concluded that the blood is deficient in viscidity and coagulability.

Where, after making repeated deep punctures, tightening the bandage round the finger, and applying pressure, we obtain only a scanty flow of blood which rapidly staunches, we may, provided always that we are not operating upon the blanched fingers of an exsanguine patient, conclude with probability that the blood is unduly coagulable.

Where the blood is so viscid that it will not enter a capillary tube, and where on flowing down over a vertical wall of glass it leaves a thick red smear behind it, the condition is one of polycythæmia.

Where the blood is pallid and flows like water the condition is one of advanced anæmia.

8. Aspect of the blood in the capsule after coagulation is complete.

Inspection of the clotted blood after the serum has separated out will furnish information which is of value both with respect to the proportion of corpuscles to blood-fluid, and with respect to the character of the blood-fluid.

After time has been allowed for complete contraction of the clot the proportion of this to the serum furnishes, as will be shown in a subsequent sub-section, an indication of the number of corpuscles in the unit volume of blood. It does so by reason of the fact that the dimensions of the contracted clot are directly dependent upon the corpuscles included in its meshes. In polycythæmia accordingly we have practically no exudation of serum from the clot, in normal blood the serum which exudes is nearly equal in bulk to the clot, and in profound anæmia the volume of the serum is many times greater than that of the clot.

With regard to the character of the serum, three points in particular may be noted:

Where bile is being absorbed into the blood—even when its quantity is much too small to give an icteric tinge to the conjunctiva, the serum shows a distinct yellow colour. We have here a very valuable indicator which tells of biliary obstruction.

Occasionally in health, after a full meal, and it would appear much more frequently in the case of Bright's disease, and also in diabetes, the serum assumes a milky appearance (Plate II., Fig. 2, *a*). This milkiness differs from that which is in question in the next sub-section, in the fact that it is due to a precipitate which is insoluble in ether, and which is so impalpably fine that it is not gathered up and held back by the very close meshes of the fibrin network (Plate II., Fig. 2, *b*).

Where through an error of technique in sealing the capsule the blood has been overheated, or where any considerable force has been

applied to the finger for the purpose of expressing the blood, the serum will often contain a trace of dissolved hæmoglobin. The mechanical hæmolysis which is in question in this last case occurs with special readiness in certain otherwise perfectly normal individuals.

9. Aspect of the blood in the capsule where agglutination of red blood corpuscles takes place under the influence of severe microbial infection.

In connexion with severe microbial infections, we have not unfrequently a development of hæmagglutinins in the blood fluids, and as a result of this, a rapid settlement of the red blood corpuscules, similar to that which occurs normally in the shed blood of the horse.* The course of events in such a blood is as follows: Very soon after the blood has been filled into the capsule, the red corpuscles run together into clumps. These clumps, which are seen to best advantage in the capillary tube which forms the recurved limb of the capsule, are at first visible only under a pocket lens. They afterwards increase in size until they are just visible to the naked eye. The corpuscles now settle down rapidly, leaving a supernatant layer of plasma (Plate II., Fig. 1, *a*). And it is to be noted that the plasma in such cases is often quite milky in appearance, by reason of its carrying in suspension enormous numbers of blood platelets.

If such a blood is looked at soon after it has been drawn off, the sedimented red corpuscles may readily be mistaken for the ordinary coagulum, and the slightly turbid or milky supernatant plasma for exuded serum.

But a little later it would be found that the supernatant fluid had resolved itself into two layers—an upper layer of absolutely limpid serum, and a lower semi-transparent or milky stratum ("buffy coat") consisting of a network of fibrin which had caught up in its meshes all the blood-platelets and minute particles which had been in suspension in the plasma (Plate II., Fig. 1, *b*).

Finally, after the lapse of some hours, the clot would differ from that of normal blood only in the respect that it was covered with a whitish pellicle, corresponding to the shrunken "buffy coat," and was studded with small prominences—each corresponding to a pellet of agglutinated corpuscles.

10. Aspect of the blood in the case where blood coagulation has been suspended by an addition of a decalcifying reagent.

Where blood coagulation is inhibited, opportunity will, of course, be given for a separation of the formed elements from the plasma

* Attention was first called by Shattock to the fact that the rapid sedimentation of the red corpuscles in horses' blood is a phenomenon of agglutination.

PLATE II.

Fig. 1. A blood capsule containing blood showing haemagglutination.

a. The blood as it appears a few minutes after the capsule has been filled.

The blood has here differentiated itself into a corpuscular deposit and a milky supernatant plasma. The agglutination of the red blood corpuscles is indicated to the eye by the rapid sedimentation, the granular appearance, and irregularity of the delimiting contour of the corpuscular deposit. The milkiness of the plasma is due to the presence of innumerable blood platelets (Chapter I., Sub-section 9).

b. The same blood as it appears a few hours later. The fibrin meshwork which has formed in the corpuscular deposit and plasma has here contracted, drawing together the former and collecting together out of the latter all the suspended particles into a "buffy" coat. In association with this a watery serum has separated itself out.

Fig. 2. Blood capsules showing blood from which a milky serum separates out (Chapter I., Sub-section 8).

a. Blood as it appears a few minutes after filling in the capsule.

b. The blood as it appears a few hours later. It will be noted that there is here no "buffy" coat and that the serum is still milky. The milkiness is thus due not as in Fig. 1, a to the suspended particles, but to elements of almost molecular fineness (Chapter I., Sub-section 8).

Figs. 3 and 4 show the results obtained in the Wassermann test (Chapter I., Sub-section 9).

Fig. 3 (a). Tubule containing a normal serum mixed with sheep's corpuscles—haemolysis.

Fig. 3 (b). Tubule containing normal serum which has been digested with a differentiating antigen, receiving afterwards an addition of sheep's corpuscles—haemolysis.

Fig. 4 (a). Tubule containing a syphilitic serum mixed with sheep's corpuscles—haemolysis.

Fig. 4 (b). Tubule containing a syphilitic serum which has been digested with the same differentiating antigen, afterwards receiving an addition of sheep's corpuscles—no haemolysis.

Fig. 5. Method of making a series of dilutions which stand to each other in the same relation as the terms in a geometrical progression (Chapter IV., Sub-section 13).

(a) shows a series of drops of diluting fluid each corresponding to one unit volume, or to two or more unit-volumes.

(b) shows the series of mixtures obtained by adding to the first drop a unit volume of the diluted (here blood), and carrying on in each case one unit-volume of the mixture from the preceding drop to the next in series.

Fig. 6 shows the appearances obtained when the blood of a leucaemic patient is allowed to settle down by gravitation after decalcification by admixture with citrate of soda (Chapter I., Sub-section 6).

Fig. 7 shows the appearances obtained when in connexion with the measurement of the coagulation-time capillary tubes which have been filled in with blood are blown out into water (Chapter IV., Sub-section 9).

a. Here coagulation has not commenced and the red corpuscles, when projected from the tube, separate out from one another forming a cloud which melts away as the corpuscles dissolve in the water.

b. Here coagulation has just commenced, and the corpuscles are collected and held together into clumps by the fine meshwork of fibrin which is beginning to form.

c. Here coagulation is complete, and a firm snake like clot has been obtained.

d. Here a snake-like clot such as was obtained in c has undergone haemolysis, and it now contains only the leucocytes of the blood and shadows of red corpuscles (Chapter IV., Sub-section 20).

PLATE II.

Fig. 1. A blood capsule containing blood showing hæmagglutination.

a. The blood as it appears a few minutes after the capsule has been filled in. The blood has here differentiated itself into a corpuscular deposit and a milky supernatant plasma. The agglutination of the red blood corpuscles is indicated to the eye by the rapid sedimentation, the granular appearance, and irregularity of the delimiting contour of the corpuscular deposit. The milkiness of the plasma is due to the presence of innumerable blood platelets (*Chapter V., Sub-section* 9).

b. The same blood as it appears a few hours later. The fibrin meshwork which has formed in the corpuscular deposit and plasma has here contracted, drawing together the former, and collecting together out of the latter all the suspended particles into a "buffy" coat. In association with this a water-clear serum has separated itself out.

Fig. 2. Blood capsules showing blood from which a milky serum separates out (*Chapter V., Sub-section* 8).

a. Blood as it appears a few minutes after filling in the capsule.

b. The blood as it appears a few hours afterwards. It will be noted that there is here no "buffy" coat and that the serum is still milky. The milkiness is thus due not as in Fig. I. (*a*) to comparatively gross suspended particles, but to elements of almost molecular fineness (*Chapter V., Sub-section* 8).

Figs. 3 and 4 show the results obtained in the Wassermann test (*Chapter X., Sub-section* 9).

Fig. 3 (*a*). Tubule containing a normal serum mixed with sheep's corpuscles—hæmolysis.

Fig. 3 (*b*). Tubule containing normal serum which has been digested with a differentiating antigen, receiving afterwards an addition of sheep's corpuscles —hæmolysis.

Fig. 4 (*a*). Tubule containing a syphilitic serum mixed with sheep's corpuscles—hæmolysis.

Fig. 4 (*b*). Tubule containing a syphilitic serum which has been digested with the same differentiating antigen, afterwards receiving an addition of sheep's corpuscles—no hæmolysis.

Fig. 5. Method of making a series of dilutions which stand to each other in the same relation as the terms in a geometrical progression (*Chapter IV., Sub-section* 13).

(*a*) shows a series of drops of diluting fluid each corresponding to one unit volume, or to two or more unit-volumes.

(*b*) shows the series of mixtures obtained by adding to the first drop a unit volume of the dilutand (here blood), and carrying on in each case one unit-volume of the mixture from the preceding drop to the next in series.

Fig. 6 shows the appearances obtained when the blood of a leucæmic patient is allowed to settle down by gravitation after decalcification by admixture with citrate of soda (*Chapter V., Sub-section* 6).

Fig. 7 shows the appearances obtained when in connexion with the measurement of the coagulation-time capillary tubes which have been filled in with blood are blown out into water (*Chapter VII., Sub-section* 9).

a. Here coagulation has not commenced and the red corpuscles, when projected from the tube, separate out from one another forming a cloud which melts away as the corpuscles dissolve in the water.

b. Here coagulation has just commenced, and the corpuscles are collected and held together into clumps by the fine meshwork of fibrin which is beginning to form.

c. Here coagulation is complete, and a firm snake-like clot has been obtained.

d. Here a snake-like clot such as was obtained in (*c*) has undergone hæmolysis, and it now contains only the leucocytes of the blood and shadows of red corpuscles (*Chapter VII., Sub-section* 20).

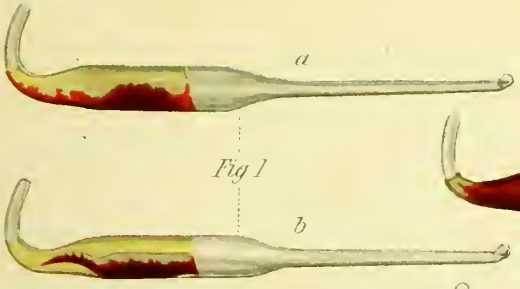


Fig 1

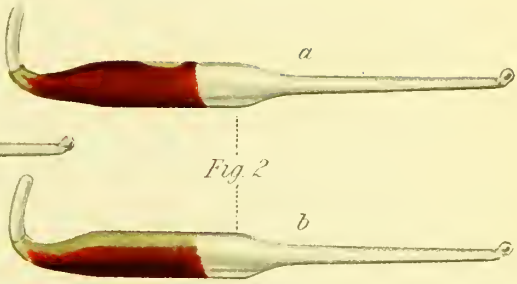


Fig 2

Fig 3.

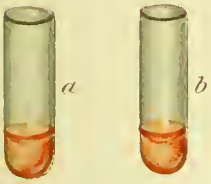


Fig 5 a.

Fig 4.

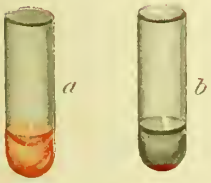


Fig 5 b

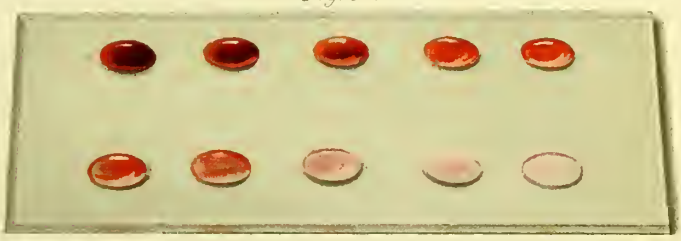
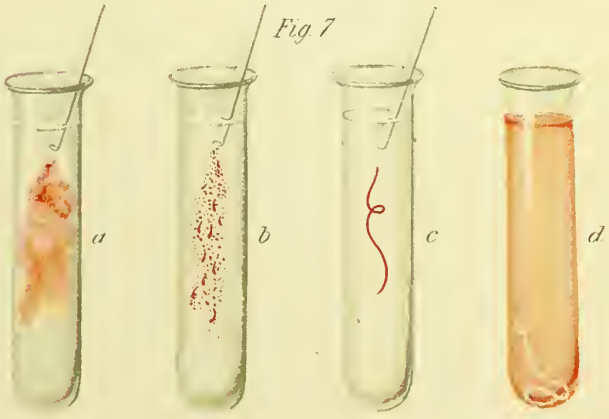


Fig 6.

Fig 7



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under the influence of gravitation. This settling down will differ from that which occurs under the influence of agglutination in the respect that it is a much slower process. In correspondence with this, there will here be a sorting out of the formed elements according to their specific gravity. In virtue of this, it will be possible, where leucæmic blood has been received into a citrate of soda solution, to make a diagnosis and to follow the progress of the case by mere naked-eye inspection. For the blood in such a case arranges itself, as shown in Plate II., Fig. 6, into three separate layers: a layer of plasma above, an intermediate thick layer of leucocytes, and below this a layer of red corpuscles.

The development of hæmagglutinins in the serum of patients who are suffering from a severe bacterial infection, has a special interest in connection with the opsonic technique, inasmuch as a serum which agglutinates the patient's own corpuscles very often agglutinates the foreign corpuscles with which it is brought into contact in testing the opsonic power of the blood.

CHAPTER VI.

METHOD OF PREPARING BLOOD-FILMS FOR MICROSCOPIC EXAMINATION.

Principles which are involved in the making of a blood-film—Conditions under which a perfectly flat film in which all the corpuscles are open to inspection can be obtained—Method of preparing a slide or cover glass for the reception of a blood-film—Method of selecting the convex face of the slide and of preparing this for the reception of a blood-film—Method of making a blood-film in which all the formed elements shall lie open to inspection, and in which the leucocytes shall, as may be required in the particular case, be uniformly distributed through the film, or collected together to the end of the film—Method of making a spreader—Advantages which attach to the smooth edge and the concave configuration of the end of the spreader—Method of preparing, from cases of extreme anæmia, films in which the corpuscles shall be as closely packed together as in those made from normal blood—Method of making a satisfactory film when we have furnished to us only a blood clot.

1. Principles which are involved in the making of a blood-film.

The essentials of a successful blood-film are (*a*) that the blood shall be spread out so flat that each corpuscle is separately displayed, and (*b*) that the leucocytes shall be disposed in the film in such a manner as to subserve the particular purpose for which the film is made. It is also, in the case where the film is spread upon a slide, desirable that it shall be spread upon the convex side of that slide (vide *infra, Sub-section* 4.)

2. Conditions under which a perfectly flat film in which all the corpuscles are open to inspection can be obtained.

A moment's thought will show that for the achievement of a flat film more is required than a spreading out of the blood over the surface of the glass. Two mutually antagonistic physical forces here come into play. We have on the one hand a force which is continually striving to pull the blood from the glass, and to mould it into a spherical shape. This is the *surface tension of the fluid.* And we have on the other hand a force which favours the dispersion of the fluid over the slide and which attaches it to the glass. We may speak of this as the force of *adhesion.*

(*a*) In the case where the surface tension overmasters adhesion, the film breaks up into a pattern of blotches and clear spaces as in Plate III., Fig. *a*. In the blotches the corpuscles are piled one on another, and in the clear spaces the surface of the glass is left bare.

(*b*) In the case where adhesion overmasters surface tension a uniform film is obtained in which we have everywhere only a single layer of corpuscles as in Plate III., Figs. *b*, *d*, *e* and *h*.

Now if the surface tension of the fluid we have to deal with were in the case of the blood, as it is in the case of mercury, immeasurably greater than the forces of adhesion, a flat film could never be obtained. The opposing forces are, however, in the case of the blood-film, so nearly equally balanced that, even when we work without foresight or thought, a certain percentage of flat films is obtained. It follows that with foresight and thought such films ought to be obtainable in a perfectly regular manner.

The following suggest themselves as possible methods of achieving this result.

(*a*) We might—following a procedure which we employ when we add a drop of alcohol to a watery bacterial suspension for the purpose of spreading it out upon glass—diminish the surface tension of the blood fluids;

Or (*b*) we might so modify the texture of the glass surface as to make it exert a greater attractive and adhesive force upon the blood fluids.

The *former* of the above suggestions may be disposed of by noting that no satisfactory method for spreading out a film by diminishing the surface tension of the blood fluids has as yet been suggested.

There remains, therefore, the device of altering the texture of the glass surface in such a manner as to increase adhesion.

3. Method of preparing a slide or cover glass for the reception of a blood-film.

It is currently believed and taught that it is the presence of grease on the slide or cover glass which causes blood to run together into blotches, and that satisfactory films cannot be obtained until such grease has been removed from the glass by prolonged boiling in caustic soda, or a mixture of sulphuric acid and bichromate of potash.

But when we note that water rounds itself into a globule when placed upon any smooth surface quite irrespectively of the presence of grease, and that it spreads out very readily over any roughened surface — ground glass for example — it becomes clear that the smoothness of the ordinary glass slide or cover glass would in itself account for the difficulties which are encountered in the making of

blood-films; and that the prolonged boiling in strong chemicals which gives us satisfactory cover-glasses might quite well operate, not by removing "grease," but by etching the glass.

Once the possibility of this occurs to the mind it immediately suggests itself that mechanical etching would be an efficient substitute for chemical etching. In point of fact this has proved to be the case, and films which are ideally flat can be obtained by spreading out the blood upon slides or cover glasses which have been roughened by rubbing with very fine emery paper (*Chapter I., Sub-section* 11).*

It will suffice to rub each slide firmly for a few seconds, and it is advisable to use small pieces of emery paper and to discard them as soon as they begin to wear smooth.

4. Method of selecting the convex face of the slide and of preparing this for the reception of a blood-film.

The first thing that has to be realised in connexion with the spreading of a blood-film upon a slide is that the ordinary microscopic slide is not flat.

If we lay it down upon the smooth surface of a tiled bench, catch it between thumb and middle finger, and try to twirl it, we shall find that it refuses to spin on the one face while it spins round easily upon the other. We thus learn that one face of the slide is concave and the other convex. Again, if we support one end of the slide upon any sharp corner—let us say upon the corner of a thick slide—we shall find that we can often rock it from side to side, showing that the slide is concavo-convex also along its transverse axis. It will practically always be found that the face that is concave along the longitudinal axis is concave also from side to side.

Consideration will now show that we ought to spread our films upon the convex face of the slide. If we employ the concave face there would—especially in the case where the slide was concave from side to side—be a difficulty in spreading out the blood satisfactorily in the trough of the slide; and again, when the slide finally comes to lie upon the stage of the microscope, it would rock under our fingers and thereby upset our focussing.

We therefore spin every slide upon the bench as we take it in hand, and, having found the side upon which it lies firm, we prepare the upper surface for the reception of our film.

As indicated above that preparation will consist in rubbing the slide with very fine emery paper.

* The scratches which are made by the emery of course disappear completely from view when filled in with cedar oil or balsam (*vide* Author's *Principles of Microscopy, Part I., Chapter III., Sub-section* 6).

5. Method of making a blood-film in which all the formed elements shall lie open to inspection, and in which the leucocytes shall, as may be required in the particular case, be uniformly distributed through the film, or collected together to the end of the film.

Where we wish to ascertain whether we have in a particular specimen of blood to deal with an excess or a deficiency in leucocytes, or where we wish to make a differential count of leucocytes, we require these to be distributed in a regular manner through the film. Where, on the contrary, we wish to pass in rapid review a large number of leucocytes, it is desirable that these should be brought together to the edge of the film.

By applying a little thought to the method of spreading the film we can arrange for the leucocytes to be distributed as we desire.

We can do so by taking advantage of the fact that the white corpuscles are spheres with a diameter of 10 μ. or more, while the red corpuscles are discs which have a diameter of 8 μ. with a maximum thickness of only 2 μ.

(*a*) *Method of making a blood-film in which the leucocytes are distributed in a uniform manner through the preparation.*—Having

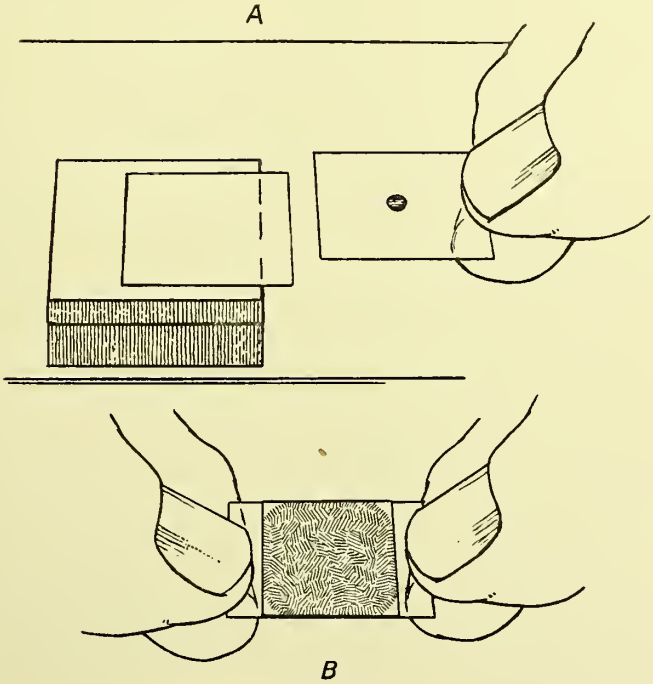


Fig. 53.

cleaned a certain number of cover glasses (cover glasses are here more suitable than microscopic slides), lay them down upon a flat slab and roughen with emery paper as described above that face of the cover glass which is to receive the film. Then remove every particle of grit. Now taking a drop of blood about the size of a large pin's head on a cover glass, turn it face downwards, and without a moment's delay impose it somewhat eccentrically upon another, pressing it down very gently.

If the surfaces are perfectly free from grit the blood will now spread out between them, and the white cells—upon which as the larger elements the pressure will have borne—will now have been flattened out and more or less firmly fixed to the glass.

As soon as this has been done, grasp without an instant's delay the two coverslips by their projecting edges, and keeping their faces parallel slide them apart by a rapid movement of both arms. The rapidity of the movement, combined with the inertia of the white corpuscles, and their adhesion to the glass ensures that their distribution on the cover-glass shall not be disturbed by this manipulation. It may, however, happen that a disproportionately larger number of leucocytes may adhere to one of the pair of cover-glasses.

In all these operations see that the steam from your fingers does not come near the films.

(*b*) *Method of making a blood-film in which the larger varieties of leucocytes are collected together at the end of the preparation.**—Place a drop of blood close to the left-hand border of a slide which is disposed with its convex face uppermost and which has been roughened with emery paper. Then having made for yourself such a spreader as is described in the next sub-section, having mastered the rationale of its construction and learned the principles which govern its use as there set forth, introduce its concave end into the drop of blood, and holding it at an appropriate angle, push it backwards and forwards within the limits of the drop, until the blood has spread out evenly in the angle between spreader and slide.

Steadying now the slide with your left hand, carry the spreader along *very slowly* from left to right. As you do so the plasma and red blood corpuscles and some of the smaller leucocytes will continuously escape through the chink between spreader and slide, while the larger

* If after making a film by the method here described we found that we wanted to use it for some other purpose—for instance, for making a differential count of the leucocytes, we could still do so by ruling a fine line with a needle-point down the middle of the slide and then counting all the leucocytes from one end of the preparation to the other, confining our attention to those which fall into the field as the eye of the microscope travels along the ruled line.

leucocytes are swept on in front until the fluid has drained away, and

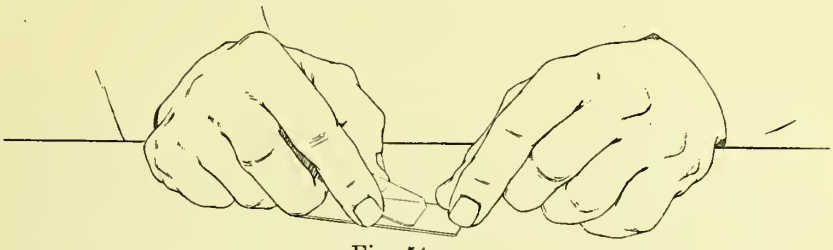


Fig. 54.

the leucocytes are stranded. These are now overtaken by the spreader and flattened out by it.

6. Method of making a spreader.*

A spreader is a glass slide broken across in such a manner as to present the appearance shown in Fig. 55. The essential points in its construction are (*a*) that its end shall be slightly concave (the concavity ought to be perfectly regular and only just appreciable to the eye) and (*b*) that its delimiting edge shall be perfectly smooth.

Fig. 55.

Provide yourself with a batch of two or three dozen slides (slides which have already been used will serve our purpose) and select for use only the thinnest ones.

Take one of these, and make a nick with the glass-cutting knife about half-way along the side. Then grasp the two ends between the fingers and thumb of either hand, and, advancing the thumb of the right hand as far, or a very little beyond, the intended line of fracture to serve as a fulcrum, break the slide across by putting a transverse strain upon it, at the same time exerting a pull in the longitudinal direction.

While the delimiting edge thus obtained, or rather, one or other of the two delimiting edges—for the edges of the obverse and reverse faces of the slide will differ from each other—will generally be ideally smooth, it is impossible to ensure that the line of fracture shall always conform to expectation. But as a general rule this will take the form of an arch, and the point which is supported by the

* The use of such a spreader was suggested by my late fellow-worker, Capt. J. Kuhnhardt, I.M.S.

thumb will correspond to the crown of the arch. Sometimes, however, no doubt by reason of internal strains in the glass, a whole box of slides will be used up before a satisfactory line of curvature has been obtained. This, as well as the character of the delimiting edges, will be judged of by turning the thin edge of the slide towards us and looking along its upper border.

When we have succeeded in satisfying ourselves, we shear off the two corners of the slide by nicking them with a glass-cutting knife; we place with a blue pencil a distinguishing mark upon the squarer of the two delimiting edges (the edge on the face which faces towards the supporting thumb will generally be flanged or undercut and unserviceable): and we then may mount the spreader on the back of another slide, fixing it there with sealing-wax.

7. Advantages which attach to the smooth edge and the concave configuration of the end of the spreader.

It may be well to set out, by way of preface, that the smoothness or roughness of the edge of the spreader determines the character of the edge of the film, and the line of curvature of the end of the spreader determines the configuration of the end of the film.

When we employ a spreader with a smooth edge we obtain in our films sharp-cut edges, such as are shown in Plate III., Figs. *d*, *e*, and *h*. Where we employ a rough edge, such as would be furnished by an ordinary slide, we obtain in our films frayed-out edges, such as are shown in Plate III., Figs. *f* and *g*. It will be appreciated that the observer who has to pass in review under the high power of the microscope a number of leucocytes which are ranged along the edge of a film obtains from the smooth, sharply cut edge exactly the same advantage as accrues to the mariner when he can follow an unindented instead of an indented coast line.

The reasons for adopting a concave configuration for the end of the spreader are the following:—

(*a*) The concavity of the spreader holds off all direct pressure from the blood-corpuscles, and enables us to adjust the vertical dimensions of chink, so as to let through the smaller formed elements of the blood, while the larger elements are carried on to the end of the spread.

It will be appreciated that as we increase or diminish the angle which the spreader makes with the slide we respectively increase and diminish the height of the arch, and so open or close down the chink, which plays the part of a strainer.

(*b*) The springers of the arch which take up the pressure and fend it off from the blood-corpuscles will function as runners when we

PLATE III

PLATE III.

This plate shows what has to be avoided and what is to be aimed at in making film-preparations of blood, and in particular film-preparations for phagocytic counts.

a. An extreme example of the unsatisfactory honeycomb film which is obtained when the surface tension of the blood fluid strips it off from some region of the slide leaving the glass bare, and piles it up on other regions until the corpuscles lie many layers deep.

b. An example of a film with a concave end. Such a film is produced when the spreader is held too upright or when its working end is too much hollowed out.

c. An example of a film drawn out into a tongue by a rectangular spreader which has also been held too obliquely.

d. Example of a film which is open to criticism only in the respect that it is carried a little too far down the slide.

f and *g*. Examples of unsatisfactory films.

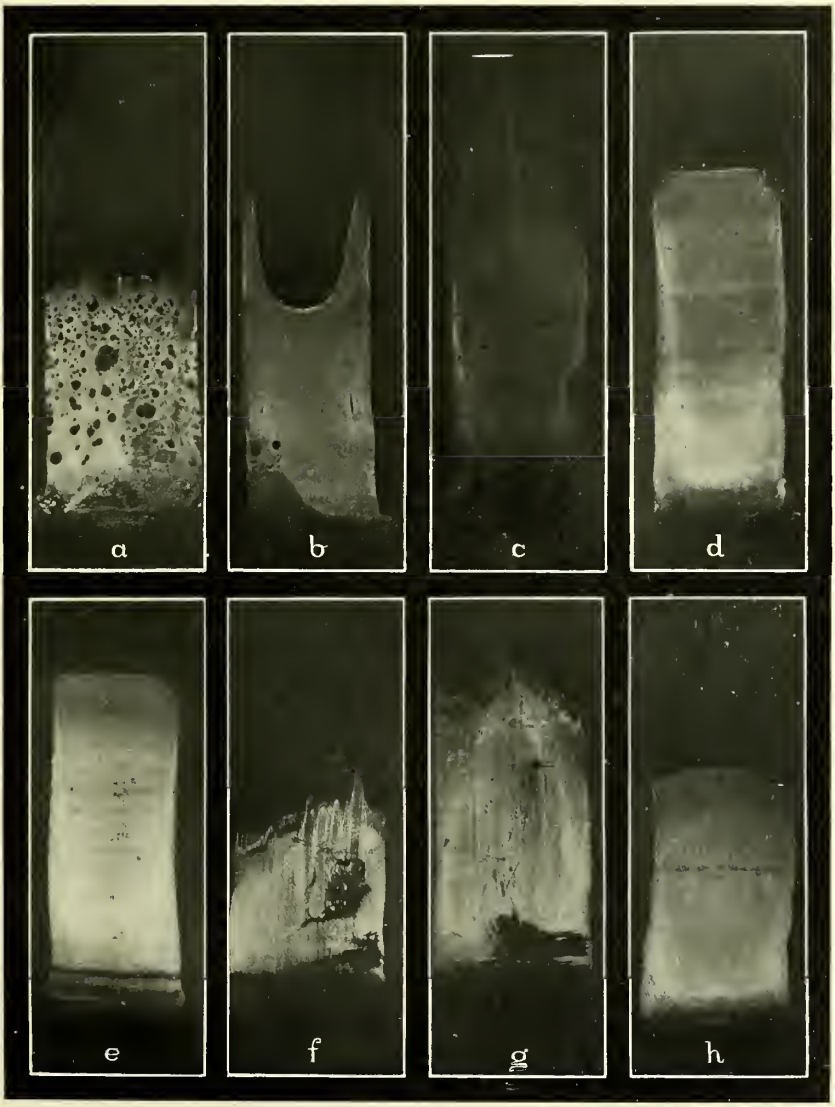
The frilled edges and striation marks which are the characteristic features of these films show that the slide which was used for spreading them had an edge toothed like a comb, and that a heavy hand brought it down upon the film, almost stripping the slide in some places, and here lacerating the leucocytes; and piling up the blood thus collected into ridges in which the leucocytes would retain their spherical shape instead of being flattened out.

These films were made by the Author of a paper on the "Inaccuracy of the Opsonic Method," and were put forward by him as satisfactory.

e. Example of a satisfactory film.

Made by the same Author after he had allowed himself to be taught.

h. A quite satisfactory film.



[*To face page 72.*

carry our hand along in making the spread. By the aid of such bearings, even when we put, as we ought, only the very lightest pressure upon them, we shall be able to keep unaltered the angle between slide and spreader, and so to obtain better films than if we were working free-hand.

(*c*) When we recall to mind that the face of the slide which we place uppermost when we set to work to make a film is raised up in the middle, it will be clear that, as compared with a square-ended spreader, a concave spreader is better adapted to give us a chink of uniform depth, and, by consequence, a film of more uniform thickness.

(*d*) In contrast to a square-ended spreader which will give us films which run out into a point, the concave spreader will give us films with square ends, and this will be a great advantage when we have to find and pass in review the leucocytes which are gathered to the end of the film.

(*e*) The reason why a spreader which has an appropriate concavity gives a square-ended film will be appreciated when we reflect that when we are coming to the end of our spread, and when in correspondence with this very little fluid blood remains over in front of the spreader, this will fail to reach the crown of the arch, with the result that the film will, along the central line of the slide, stop short at this point. It will, however, still be carried forward by the wings of the arch, which are following on a little way behind.

Now, if the arch is as it should be—a very flat arch—the film will be carried only a very little further, only, in fact, until the lateral portion of the film has been brought up into a line with the central portion.

If, however, the arch of the spreader has too great a rise, or if the spreader is held too upright, the blood will—as experiment and reflection will show—be carried on too far by the wings of the arch, with the result that an undesirable concave configuration will be given to the film (Plate III., Fig. *b*).

8. Method of preparing, from cases of extreme anæmia, films in which the corpuscles shall be as closely packed together as in those made from normal blood.

After obtaining uncoagulated blood in the manner described in *Chapter V., Sub-section* 6, seal up the tip of the capillary stem, leaving an air space between the column of blood and the end of the pipette. Then place this upright, so as to allow of the sedimentation of the corpuscles. When these have settled, pass down into the stem of the pipette a finer capillary pipette and draw off the supernatant

fluid, leaving behind about an equal bulk of corpuscles and fluid. Then break off the tip of the capillary stem and mix the corpuscles thoroughly with the fluid; and spread films in the ordinary way.

9. Method of making a satisfactory film when we have furnished to us only a blood clot.

Shake up the blood clot in physiological salt solution, pipette off this fluid from the clot, allow the corpuscles it contains to sediment, and then proceed as in the foregoing sub-section.

CHAPTER VII.

METHODS OF BLOOD TESTING, OTHER THAN THOSE WHICH ARE DIRECTED TO THE MEASUREMENT OF ITS BACTERIOTROPIC POWERS.

SECTION I.—METHOD OF MEASURING THE PROPORTION OF CORPUSCLES TO FLUID IN A SAMPLE OF BLOOD.

1. Preliminary elucidation.

The measurement of the proportion of corpuscles to fluid serves exactly the same purposes as the enumeration of the corpuscles in

the unit-volume of blood. The normal proportion of corpuscles to fluid is about 55 volumes per cent.

2. Method of carrying out the measurement where we can draw off the blood directly from the patient.

Principle of the method.—Measured volumes of blood, drawn off from the finger, and of a citrate of soda solution are mixed together and are drawn up into a graduated hæmocrit pipette. As soon as settlement is complete the proportion of corpuscles to fluid is read off.

Requisites for carrying out the method.—A hæmocrit pipette provided with a distal throttle and graduated, as described in *Chapter III., Sub-section* 8; a tightly fitting rubber teat; a 6 to 8% solution of citrate of soda; and a slide and watch-glass.

Working instructions.—Pour out a little of the citrate of soda solution into a watch-glass, fit the rubber teat to the hæmocrit pipette, and then draw up into this three unit-volumes of the solution and transfer these to a slide. Then puncture the patient's finger near the nail on its upper surface and press out a large drop of blood, and now aspirate into the pipette as many complete volumes of blood as are at disposal in the drop which has been pressed out from the patient's finger. Where more than six volumes of blood are at disposal we must—since we have only six divisions on the stem of the pipette—break the column after drawing up the six volumes and interpose there a bubble of air. Having obtained, either at a single operation or in a series of instalments, in all 15 volumes of blood, mix them without delay with the three volumes of citrate upon the slide and then draw up into the pipette 12 volumes of the mixture. Keeping the distal end of the column of blood closely applied to the extremity of the capillary stem we seal up the throttle in a peep-flame.

Where there is no special hurry we set up the pipette on end in a rack and leave it to sediment for 24 hours or more (the period required for quite complete sedimentation will of course vary with the fineness of the capillary stem) and then read off the volume of the corpuscles upon the scale (Frontispiece, Fig. 3).

Where the result is required at the end of a few hours, we set up the pipette on end as before, and when settlement has progressed up to a certain point cut across the stem of the pipette just above the level to which the corpuscles have settled down, and then place the amputated end in the centrifuge.

Where the result is required with the minimum of delay we use a very stumpy pipette and cut it across, immediately after filling in, at the upper delimiting mark and place it in the centrifuge.

3. Method of carrying out the measurement where we have at disposal only a sample of the coagulated blood in a capsule.

Preliminary elucidation.—Inasmuch as the fibrin which can be obtained from blood corresponds to only about 0·3% of its volume, the proportion of serum to clot is, as soon as contraction has taken place, sensibly the same as the proportion of corpuscles to plasma in the uncoagulated blood.

Principle of the method.—After all the serum has been expressed from the clot, we measure this by drawing it up into a simple capillary pipette which has been furnished with a fiduciary mark. We now empty out the clot, and then measure it by filling in out of the same pipette as many unit-volumes of fluid as are required to fill up the space it occupied.

Requisites for carrying out the method.—We require only a simple capillary pipette.

Working instructions.—Hang the blood capsule into the bucket of the centrifuge by its recurved limb; centrifuge it until all the serum has been expressed from the clot; cut off the recurved end of the capsule; place a mark upon the barrel at the level of the upper margin of the serum; and, if we have to deal with a conical clot, such as will have been formed if blood occupied when it was clotting the conical end of the capsule, place another mark at the level corresponding to the upper end of the clot.

Now, having first estimated with the eye the amount of serum which has to be dealt with, and having considered the question as to what degree of accuracy in measurement is required, place a division mark upon the stem of a simple capillary pipette, choosing for it a position according to the requirements of the particular case. Where dealing with an estimated volume of 80 to 100 cmm. of serum, give such a position to the fiduciary mark as will give us a unit-volume of about 5 cmm.; where we have less serum than this aim at a unit-volume of 2 to 3 cmm. And, in any case, it will be convenient and will accelerate work if, by the technique described in *Chapter IV., Sub-section* 17, a series of further division marks are placed upon the stem so as to allow of more than one unit-volume being measured off at once.

Now introduce the measuring pipette into the serum and aspirate into it every particle of serum, dividing off successive unit-volumes, or, as the case may be, successive groups of unit-volumes by air bubbles, keeping careful tally as you do so, and letting the serum as you proceed run up into the barrel of the pipette.

In the case where you have a conical clot, and where this has moulded itself to the wall of the capsule, the serum will not, for the

purposes of the measurement, be further required. But it must be carefully reserved where the clot has not applied itself closely to the wall. The operation of measuring the serum completed, the next step will in each case be to empty out the clot.

If it has been practicable to delimit accurately with a pencil mark the space occupied by the clot, it can then immediately be filled in with fluid by the aid of the graduated pipette, keeping of course accurate tally of the number of unit-volumes which are being added.

Where the clot did not mould itself to the walls we reintroduce into the emptied capsule the serum which was drawn off, and now make a pencil line to mark its upper limit.

It will be clear that the space between this division mark and the upper division mark will correspond in volume to the clot.

The cubic capacity of this space in terms of the particular unit-volumes which here come into question is now measured by filling into it out of the pipette whatever number of unit-volumes of salt solution may be required.

Method of working out the results.—These are worked out in either case by a simple proportion sum—

Volume of serum + volume of clot : volume of serum :: 100 volumes of blood : percentage volume of serum.

EXAMPLE.—Number of unit-volumes of clear serum pipetted off from clot $25\frac{1}{2}$ (circ.)
Number of unit-volumes contained in space occupied by clot ... 23.
$48\frac{1}{2}$ (circ.) : $25\frac{1}{2}$ (circ.) : : 100 : *Answer.*

ANSWER.—Approximately 52·5 volumes per cent. of serum.

SECTION II.—METHOD OF MEASURING THE COAGULATION-TIME OF THE BLOOD.

4. Principle of the method.

A number of samples of blood are filled in at one and the same time into a series of capillary tubes and are maintained at a standard temperature.

The condition of the blood in these is investigated at progressive intervals and the minimum time required for the production of a clot is in this way elicited. This is spoken of as the *coagulation-time.*

5. Preliminary considerations.

The detailed description of the method may be prefaced by the consideration of certain questions which, though they are important, do not touch the principle of the method. These have reference to (*a*) the selection of the standard temperature, (*b*) the selection of the end point phenomenon, and (*c*) the fallacies which have to be guarded against.

(*a*) *Question as to the most suitable standard temperature.*—There are two temperatures which suggest themselves as suitable standard temperatures for the purpose we have here in view. One is the temperature of "half blood heat" (18·5° C., approximately 65° F.), which was originally selected by me for this purpose; and the other is the temperature of the blood (37° C.), which I adopted a few years later.

The former temperature has an advantage in the respect that, inasmuch as it conforms fairly closely in temperate climates with the ordinary temperature of the hospital ward and laboratory, we are relieved from the necessity of maintaining an artificial temperature. On the other hand, the adoption of "half blood heat" as a standard temperature is wasteful of time, for the coagulation time of normal blood in a capillary tube ranges between 5 and 10 minutes, and in the case of hæmophilic blood it may exceed an hour. Taking not only this into consideration, but also the improvements in the technique which facilitate rapid working, we shall do well definitely to adopt 37° C. as our standard temperature.

(*b*) *Question as to what is the best end-point phenomenon.*—Where we are blowing out capillary coagulation tubes we may adjudge coagulation to have occurred (*a*) when the blood has clotted so firmly in the tube as to make it impossible to blow it out (Author); (*b*) when on breaking across the tube and separating the fragments a filament of clot comes into view (Sabrasez); (*c*) when on blowing out the blood upon filter paper (Author) or into water (Hingston-Fox) a thread of clot is brought into view; or (*d*) when after a throttle has been formed upon the end of the capillary tube, it is found on attempting to blow out the tube that that throttle has become blocked with clot (Collingwood).

In the case where we are dealing with normal blood we can avail ourselves of any one of these end-point phenomena. In the case, however, of feebly clotting bloods—in certain hæmophilic bloods for instance—the clot may never become sufficiently firm to hold together in the form of a filament, nor acquire sufficient solidity to block the tube. In such cases we must either go to the inconvenience of throttling our tubes, or blow out the contents of our tubes on to filter paper or into water.

The last-mentioned method gives the finest differentiation, and it is therefore adopted in the technique described below.

6. Fallacies which have to be guarded against in measuring the coagulation-time of the blood.

The discussion of the fallacies which are incident to the measurement of the coagulation-time of the blood cannot be properly appreciated

until it has been clearly realised that the coagulability of the blood is profoundly influenced both by the external and by the internal respiratory interchange, and that it increases with the amount of CO_2 which is carried by the blood. This accounts for the fact that the blood in the right is more coagulable than in the left heart; and that the venous blood in a region where metabolism is active is more coagulable than that in a region where metabolism is less active.* The rate of blood clotting is thus a function which varies from moment to moment, and which has direct reference only to the blood in the particular vascular area from which the specimen has been drawn and to the particular conditions under which it has been taken.

It must, therefore, be clearly understood in connexion with the procedure described below, that what we here aim at measuring is the coagulation-time of blood obtained as unmixed as possible from the capillaries of the finger pulp under conditions of circulation and internal and external respiration which are as nearly as possible normal.

We may group the fallacies which are incident to any procedure for the measurement of blood coagulation under the following headings :— (*a*) Fallacies due to disturbance of the external respiratory interchange ; (*b*) fallacies due to disturbance of the internal respiratory interchange in the vascular area from which the blood is drawn; (*c*) fallacies due to the admixture of extraneous elements with the blood which comes under examination; (*d*) special fallacies incident to the particular procedure selected for measuring the coagulation-time.

(*a*) *Fallacies of the disturbance of the general respiratory interchange.*—Under this heading we have to note the fallacy of increased ventilation of the blood, and the fallacy of diminished ventilation. Increased ventilation of the blood, such as is obtained by deeper or more rapid respiration, protracts the coagulation time of the blood. Holding the breath, provided always that it is not held too long, shortens it. It was pointed out by Wooldridge that a general increase in the coagulability of the blood is obtained in connexion with the ingestion and absorption of food, and in particular in connexion with the ingestion and absorption of fatty food.

(*b*) *Fallacy of the disturbance of the respiratory interchange in the particular vascular area from which blood is drawn.*—Where the muscles

* Wright, on the conditions which determine the distribution of the coagulation following the intra-vascular injection of a solution of Wooldridge's tissue-fibrinogen. "Journal of Physiology," vol. xii., No. 2, 1891.

Wright.—A study of the intra-vascular coagulation produced by the injection of Wooldridge's tissue-fibrinogen.—"Proceedings Royal Irish Academy," 3rd Series, Vol. ii. No. 2, 1891

Wright.—Influence of carbonic acid and oxygen upon the coagulability of the blood *in vivo*.—"Proceedings Royal Society," Vol. lv.

of a limb are thrown into activity,* or where a ligature is applied round a limb in such a way as to obstruct the circulation, and is maintained there for more than a few seconds, the coagulation-time of the blood in that limb is accelerated. We have here an important but an avoidable source of fallacy.

(c) *Fallacy of the admixture of extraneous elements with the blood under examination.*

An *admixture of lymph or tissue-fluids to blood*—and we may assume that such admixture occurs when pressure is applied to press out blood from the punctured finger—accelerates blood coagulation.† We have here the most important and insidious fallacy of blood coagulation estimations which could completely be eliminated only by drawing off blood directly from one of the larger blood-vessels. Where, as in the method here employed, blood is obtained from a puncture into the tissues, all that it is possible to do is to make a free puncture, to apply in all cases an absolute minimum of mechanical pressure; and when we have obtained a really satisfactory outflow of blood to take full advantage of this by filling in from it, instead of only one coagulation tube, a whole number simultaneously. Provision for doing this is made in the method described below. A further point to bear in mind here is that inasmuch as the fallacy which we are here considering operates always in the direction of making us underestimate the coagulation-time it will be well, where two estimations of the coagulation differ, to decide always in favour of the one which gives the longer coagulation-time.

An admixture of elements derived from coagulated blood accelerates coagulation.—This fallacy would come in if successive samples of blood were drawn off from the same finger without careful intermediate washing. The best way of avoiding it is always to use another finger when drawing off a fresh quantum of blood.

An admixture of water with the blood accelerates coagulation.—This fallacy may come in if the finger from which blood is drawn off is not perfectly dry; and of course it may also come in if water should find access to the coagulation tubes.

(d) *Fallacies which may creep in in connexion with the technique.*—The two main issues which here suggest themselves are, first, the question as to how far *variations in the calibre of the coagulation tubes*, and secondly, the question as to how far *divergences from the standard temperature* affect the coagulation-time.

* *Wright.*—"Journal of Physiology," loc. cit.

† *Wright.*—On the effect exerted upon the coagulability of the blood by an admixture of lymph.—"Journal of Physiology," Vol. xxviii., No. 6, 1902.

On putting the matter to the test of experiment, my fellow-worker, Dr. Parry Morgan, and I myself have satisfied ourselves that within comparatively wide limits, differences of calibre in capillary coagulation tubes do not sensibly affect the result.

Dr. Dale has arrived at very similar conclusions with respect to temperature. When working with the standard temperature of 37° C., a divergence of two degrees Centigrade on either side of that temperature is permissible.

Finally, there has to be considered the possibility of error creeping into our measurement through *lost time in sealing up the tubes* and getting them into the water-bath after they have been filled in with blood. The only way to provide against this possibility of error is to find out exactly what time one requires for the operations in question (in connexion with the technique described below an interval of twenty seconds will be found amply sufficient) and then to delay in every case the transference of the tubes to the water-bath until this time limit has expired.

7. Requisites for carrying out the method.

A many-stemmed pipette, made as described in *Chapter III., Sub-section* 7, and furnished with a closely-fitting rubber teat; a cylindrical glass vessel which will hold two or more litres of water (or, where such is not at hand, any large vessel to serve as a water-bath, and, in addition, any shallow or transparent vessel which will permit of our seeing what happens when we blow out our tubes into water); a thermometer and plasticine with which to suspend it from the wall of the water-bath; a Bunsen flame or a spirit lamp for sealing up our tubes; a pricker and bandage for obtaining blood from the finger; a stop-watch or watch with a second hand; and paper and pencil for making notes.

8. Working instructions.

Having fixed our thermometer on to the wall of our water-bath, and added hot water until the thermometer stands at 37° C., we place our many-stemmed pipette ready to hand, taking care first to make gentle pressure upon the plasticine pellet, so as to separate out our capillary tubes sufficiently to see what goes on in them.

This done we prick deeply into the finger, and apply our bandage, making only very light pressure. If when this is done the blood does not well up as freely as we wish, we go on to another finger, make a deeper puncture, or two or three punctures, and do not rest satisfied until we have obtained a copious supply of blood direct from the capillaries, free from admixture with lymph.

We now establish a negative pressure in the interior of the teat, and, bearing in mind that we have to fill in here only the four longer tubes and not the by-pass, we introduce into the blood as it flows from the finger, first the end of one tube, and then the ends of the other three in succession; or, if this comes easier, the ends of two, three, or four tubes at a time. In this latter case we should find that, with capillary stems of at all the same calibre, sensibly the same amounts of blood would be aspirated into each. In the former case it would, on removing the orifice of one capillary stem from the blood to aspirate through another, be found that the column of blood in the tube which had been filled in would remain stationary until every tube had been filled up to the same extent.

Fig. 56.

Having filled in our tubes in this way (Fig. 56), we set the stop-watch going, or get an assistant to read off and note down the time. While this is happening we turn up the points of the capillary stems so that the blood may run down and leave the ends of the tubes free for sealing, and at the same time we relax the grasp of our fingers upon the teat, and draw in air through the by-pass to abolish the negative pressure.

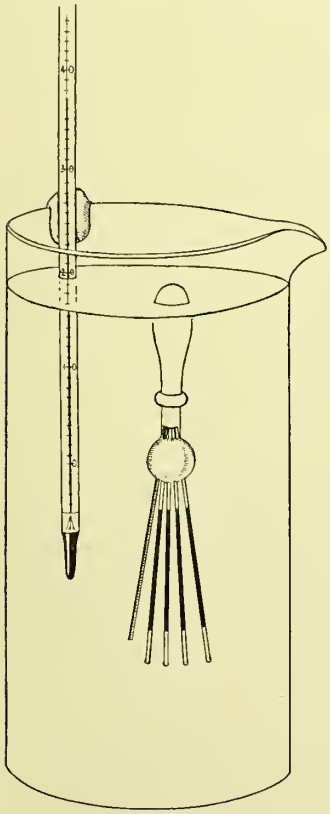


Fig. 57.

We now introduce the ends of the whole four tubes simultaneously into the flame of a Bunsen burner, and, as the walls of our capillary tubes are very thin, and the blood has been got well out of the way, the sealing will usually occupy only a few seconds.

All this while we are keeping a watch upon the time, and when the interval which has been allotted for *lost time* (*vide Sub-section* 6, *d*, *supra*) has elapsed, we transfer our pipette to the water-bath, floating it upon the water as shown in Fig. 57.

On sitting down to watch our pipette we shall see that, in addition to the help which the by-pass gives in connexion with the sealing of the tubes, it performs

a further useful office in connexion with the opening up of the tubes. As the air in the teat expands in the warm water we see bubbles begin to issue from the by-pass. And it is clear that if there was not this provision for their escape, there would have been generated in the pipette a positive pressure, which would have interfered with the deliberate opening up and blowing out of the tubes.

And yet a further point comes up for consideration. It is clear that if we were now to open up one of the tubes which contains a column of blood, and to make pressure upon the teat, there would be such free vent for the air through the by-pass that it would not be possible to bring any appreciable expulsive force to bear upon the column of blood in the tube which we have opened up. It follows that, before going to work to blow out a blood-tube, we must introduce a resistance into the by-pass tube. We can do this very simply. As soon as the issue of bubbles from the by-pass has completely ceased, we compress the teat to almost infinitesimal extent, and then let go. The compression which is applied suffices to drive out the column of air which occupies the by-pass tube, and when we let go this column is replaced by a column of water (*vide* Fig. 57). This provides the resistance we require, and it is clear that if, in lieu of being merely trimmed off, the by-pass tube had been cut off short, this resistance would not have been adequate.

Everything is now ready for testing the condition of the blood in the first tube as soon as the proper interval shall have elapsed. In the case of an ordinary blood we may wait $1\frac{1}{2}$ to $1\frac{3}{4}$ minutes from the time of filling in. Taking hold of the pipette by the barrel we raise it out of the water for an instant, break off with finger and thumb the tip of the first capillary tube, and then dipping it into the water-bath (or into the vessel which replaces it for this purpose) we blow out the tube by forcibly and suddenly compressing the teat. We blow out the rest of the tubes in the same way at $\frac{1}{4}$ minute to $\frac{1}{2}$ minute intervals.

If in the course of these operations the teat and barrel have filled with water, we may find it convenient to suspend the pipette from the wall of the water-bath, fixing it there by plasticine as was done at the outset with the thermometer. If on the other hand we have restored the buoyancy of the pipette by blowing out the water, we must before dealing with the next blood-tube, fill in all open tubes with water.

9. Method of reading off the results.

The appearances which present themselves on blowing out the coagulation tubes are indicated in Fig. 58, A, B and C, and are very perfectly shown in Plate II., Fig. 7.

Picture obtained when coagulation has not yet begun.—Here, if the contents of the tube are projected with sufficient force, the red corpuscles disseminate themselves through the water in the form of a fine cloud, which melts away like a mist as the corpuscles dissolve, Plate II., Fig. 7, *a*.

But if the contents of the tube are not projected with sufficient force, the blood will naturally sink to the bottom in a shower of drops and the appearances will be indistinguishable from those obtained in the next case.

Picture where coagulation is just commencing.—We have here a shower of granules, the corpuscles being held together into clumps by the fine meshwork of fibrin which is just beginning to form, Plate II., Fig. 7, *b*.

Picture obtained when coagulation is complete.—Here a firm cylinder of clotted blood is blown out of the tube. It generally sinks to the bottom, but it will sometimes float up to the surface of the water, Plate II., Fig. 7, *c*.

When coagulation is not quite so complete, we obtain, instead of a clean firm cylinder, a wisp of clot which drops to the bottom, leaving as it sinks a trail of blood corpuscles behind.

For the purposes of our measurement we may adjudge coagulation to have occurred as soon as anything in the nature of a cylinder of clot makes its appearance.

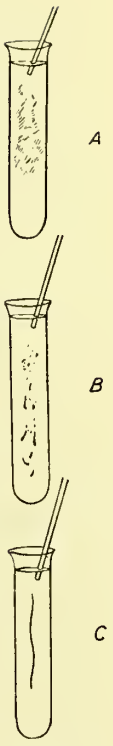


Fig. 58.

SECTION III.—METHOD OF MEASURING THE CONTENT OF THE BLOOD IN CALCIUM AND MAGNESIUM SALTS.

10. Preliminary elucidation.

Where calcium or magnesium salts, or citric acid, have been administered, this method enables us to ascertain how much has been absorbed into the blood.

11. Principle of the method.

To a series of precisely similar volumes of blood is added a series of similar volumes of progressively diminishing concentrations of a decalcifying agent. A specimen sample of each mixture is then observed with a view to ascertaining what is the minimum concentration of the reagent which suffices to prevent clotting.

12. Requisites for carrying out the method.

A throttled composite pipette fitted with a tightly-fitting rubber teat; a standard solution of oxalate of ammonium; physiological salt solution made with chemically pure salt; watch glasses for

containing these solutions; and smooth glass slides upon which the dilutions and mixtures can be made.

13. Working instructions.

Make up a stock solution of oxalate of ammonium in physiological salt solution by weighing out 1·755 grammes of oxalate of ammonium and 8·5 grammes of chemically pure sodium chloride and dissolving it in 1 litre of distilled water. The solution of oxalate of ammonium thus obtained will be a $\frac{N}{80}$ solution, and must be kept in the dark.

Using physiological salt solution as a diluent, make from this by the technique described in *Chapter IV., Sub-section* 12, an $\frac{N}{100}$, $\frac{N}{120}$, $\frac{N}{150}$, $\frac{N}{180}$, $\frac{N}{200}$, $\frac{N}{250}$, and $\frac{N}{320}$ dilution, making the $\frac{N}{100}$, $\frac{N}{120}$, $\frac{N}{200}$, $\frac{N}{240}$, and $\frac{N}{320}$, dilutions directly from the stock solution; the $\frac{N}{150}$ from the $\frac{N}{100}$; and the $\frac{N}{180}$ from the $\frac{N}{120}$. Arrange these dilutions as they are made in a series of consecutive drops on one or more glass slides, mixing in each case thoroughly, and spacing out the drops sufficiently to prevent their running into each other.* Also take care, where secondary dilutions are to be made from a primary dilution, to employ in making that primary dilution sufficiently large unit-volumes of fluid to leave over afterwards the necessary surplus.

Then placing a fiduciary mark on the stem of the throttled pipette at any convenient point, transfer—taking the precautions insisted on in *Chapter IV., Sub-section* 7—a unit-volume of each dilution to a clean slide, arranging the drops in order and remembering, if you are using a pipette with a proximal throttle, never from start to finish to relax the pressure on the teat sufficiently to allow of any fluid passing up into the throttle.

This done, prick the finger and press out a large drop of blood, and now using the same fiduciary mark as before, and dividing off, of course, with bubbles of air, aspirate into the throttled pipette a series of unit-volumes of blood corresponding to the series of drops which is ranged upon the slide.

Having measured off the required number of volumes of blood—and it is not essential that they should all be obtained from a single drop of blood—these are to be blown out one by one, each into one of the drops of the decalcifying fluid. When this operation is complete go

* They will have very little tendency to do this if the surface of our slide is quite smooth.

back over the whole series of drops, beginning with that which contains the weakest oxalate solution, carefully mixing in each case by aspirating into and expelling from the pipette.

When this has been done a specimen sample of each drop—conveniently such a sample as will fill in the capillary pipette as far as the fiduciary mark—is to be aspirated into the stem of the pipette, and the point is then sealed in the flame. The pipette is then laid upon its side in an incubator until coagulation is complete. It is then set upright to allow of the settling down of the corpuscles in the unclotted unit-volumes of blood.

In the ordinary case a $\frac{N}{180}$ or $\frac{N}{200}$ solution of ammonium oxalate will be the weakest solution which will inhibit coagulation.

14. Points to which observation is to be directed in reading off the results.

The results can generally be read off by the end of an hour, or even before that. But, owing to the fact that clotting may be very long delayed where nearly all the calcium has been precipitated, the results cannot be taken as absolutely final before the expiration of twelve hours.

The more important of the differences upon which we may rely in judging whether a sample of blood has or has not clotted will be learned on turning to the Frontispiece, Fig. 2.

Where clotting has been completely prevented we have a layer of corpuscles filling in the whole diameter of the tube, and above this a layer of plasma also occupying the whole width of the tube, and the dividing line between these is a straight horizontal line.

Where we have a clot, the serum, as it separates out, intervenes between the clot—or, at any rate, between the upper portion of the clot—and the wall of the tube.

In exceptional cases, where the clot is very delicate, and where the reading off of the results has been unduly postponed, this difference between the clotted and unclotted samples may be effaced by the settling down of the clot. All that it will be necessary to do in such a case to bring out the difference will be to turn the pipette upside down for a few minutes.

The following table will be found useful :—

$\frac{N}{80}$	sol. contains	1·755	parts of	ammonium	oxalate	in 1,000, equivalent to	0·7	parts CaO. in 1,000.
$\frac{N}{100}$	,,	1·4	,,	,,	,,	,, ..	0·56	,,
$\frac{N}{120}$	,,	1·17	,,	,,	,,	,, ..	0·46	,,

$\frac{N}{150}$	sol.	contains	0·93	parts	of	ammonium	oxalate	in 1,000, equivalent to	0·37	parts CaO. in 1,000.
$\frac{N}{180}$	,,	,,	0·78	,,	,,	,,	,,	..	0·31	,,
$\frac{N}{200}$	,,	,,	0·7	,,	,,	,,	,,	..	0·28	,,
$\frac{N}{240}$	,,	,,	0·58	,,	,,	,,	,,	..	0·23	,,
$\frac{N}{320}$	,,	,,	0·43	,,	,,	,,	,,	..	0·17	,,

SECTION IV.—METHOD OF MEASURING THE ALKALINITY OF THE BLOOD.

15. Preliminary elucidation.

(*a*) *Clinical purport.*—This method enables us to detect conditions of acid-intoxication.

Such a condition is one of the most serious complications of diabetes, being the precursor and accompaniment of diabetic coma.

A condition of well-marked acid-intoxication has been found in association with typical cases of scurvy in adults.* It has been found also in connexion with every one of the few cases of infantile scurvy which have come under the observation of the author. And, lastly, it has been found by the author in children who were suffering from serious malnutrition, and who were being fed on a scorbutic dietary, but who showed no overt signs of scurvy.

(*b*) *Precise scope and object of the investigation.*—What we desire to ascertain by a measurement of the alkalinity of the blood fluids is whether these contain a sufficiency of bases in loose chemical combination to supply the needs of the tissues, to carry off the CO_2 to the lungs, and to subserve whatever other purposes loosely combined bases may subserve in connexion with the metabolism of the body.

The indicator required for such a measurement is a reagent possessing the chemical affinities of an acid (*a*) which would, by a distinct colour change, tell us when it unites with a base, (*b*) which would enter into a combination with a base when free or conjugated with a very weak acid like carbonic acid, but (*c*) which would not be able to possess itself of the base when conjugated with a strong acid.

Red litmus satisfies these requirements. We may conceive of red litmus as a weak acid—"litmic acid"; and of blue litmus as a "litmate" or salt of that acid. In accordance with this, when red

* *Wright*—On the pathology and therapeutics of scurvy.—*Lancet*, August 25, 1900.

litmus changes to blue in the presence of a free base, we may conceive of this as a combination of free acid with free base; when the same colour change occurs in the presence of an "alkaline salt," such as a carbonate, we may conceive of it as being due to the transfer of a base from a weaker to a stronger acid; and when the litmus remains red in the presence of a neutral salt like sodium chloride or an acid salt like $Na\ H_2\ PO_4$, we may conceive of the litmic acid as remaining uncombined owing to its being incompetent to oust the hydrochloric acid or, as the case may be, the phosphoric acid from its combination with the base.

Conceiving in this manner of the *rôle* played by the indicator in our titrations of blood fluids *in vitro*, we see that it may be compared with that which would be played in these fluids *in vivo* by a cell which is hungry for bases.

16. Principle of the method.

A series of small volumes of serum are titrated in a capillary pipette with a series of similar volumes of progressively increasing dilutions of a standardised sulphuric acid, the resulting mixtures being in each case tested with red litmus paper to ascertain what is the minimum dilution of the standard acid which abolishes the alkaline reaction of the serum.

17. Requisites for carrying out the method.

Blood filled into a capsule made from a glass which is free from alkali; capillary pipettes made from such glass; a tightly-fitting rubber teat; a very sensitive red litmus paper; deci-normal sulphuric acid; neutral physiological salt solution to serve as a diluting fluid; and watch-glasses and slides for holding the solutions, and making the dilutions and mixtures. Among these requirements the most important, from the point of view of the avoidance of fallacy, are: (*a*) the requirement that glass which is employed, both for the blood-capsule and the pipette, shall be free from alkali; and (*b*) the requirement relative to the sensitiveness of the litmus paper.

The question of the alkalinity of the glass has already been treated of in *Chapter I., Sub-section* 2, and provision for the detection of any alkali from this source is made in the working instructions set forth below.

The question of what is required in the test-paper may be dealt with briefly here. Premising that the alkalinity of the normal serum, plasma and lymph is $\frac{N}{35}$, it is clear that if we hope to record a 10%

divergence from this normal, we must have at disposal a red litmus paper which will turn blue in a $\frac{N}{350}$ alkaline solution.

In order to see whether our litmus paper comes up to this standard, we may make for ourselves a $\frac{N}{350}$ alkaline solution by dissolving 0·8 gramme of crystallised carbonate of soda in 1 litre of distilled water, or more simply we may make for ourselves such a test-fluid by taking up into a capillary pipette one unit-volume of serum and nine unit-volumes of physiological salt solution.

If we get a distinct blueing of our litmus paper with these test-fluids, we may be satisfied.

If on the other hand we fail to get a distinct colour change, our litmus paper is not sufficiently sensitive, and we must provide ourselves with a more sensitive paper.

We can easily make such a test-paper for ourselves if, setting out with the best blue litmus paper we can procure, and bearing in mind that a defect of sensitiveness in a red paper must be due to a residue of free acid left behind in the fabric, we turn the blue litmus paper red with an absolutely minimum addition of acid, and then wash it out by prolonged soaking in many changes of distilled water.

18. Working instructions.

Begin in each case by testing the litmus paper and the pipettes. The testing of the former has just been described; the latter may be tested by drawing up into them a minute quantity of distilled water, making this traverse the whole length of the stem, and then blowing it out upon a piece of sensitive red litmus paper. If the glass is of satisfactory quality there should be no blueing of the litmus. These points having been set at rest, pour out into a watch-glass a small quantity of the deci-normal sulphuric acid, and into another a larger quantity of physiological salt solution; then, having laid down in front of these a couple of clean glass slides, take in hands a capillary pipette furnished with a division mark about 1·5 to 2 cm. from its orifice. Now proceed to make from the deci-normal acid by the technique described in *Chapter IV., Sub-section* 12, a $\frac{N}{30}$, a $\frac{N}{35}$ and a $\frac{N}{40}$ solution, *i.e.*, a 3-fold, a $\frac{2}{7}$th, and 4-fold dilution, and range these in successive drops on one of the glass slides.

Now lay ready to hand a slip of red litmus paper, and break off the end of the blood-capsule in such a way as to get at the serum without mixing it up with blood corpuscles, and then using a very fine capillary pipette in order to economise serum and to secure great

accuracy of measurement, draw up into it first one unit-volume of serum, and then one unit-volume of $\frac{N}{30}$ acid. After blowing out and mixing these together carefully on a slide, transfer a drop of the mixture to, let us say, the left-hand end of the slip of litmus paper. Repeat the same operation with the $\frac{N}{35}$ and with the $\frac{N}{40}$ acid. If we are employing an unglazed litmus paper, the results will be immediately visible; if we are using a glazed litmus paper they will be visible only after the lapse of a minute or two, and will be best seen after the drop of fluid has been sucked off by a pipette; and in either case the reading off of the results must not be delayed till the test-paper has become dry.

In the case where we are dealing with a normal serum, it will be found that mixture of serum with the $\frac{N}{30}$ acid leaves the colour of the test-paper unaltered; that the mixture of serum with the $\frac{N}{35}$ gives a distinct but not deep blue; and that the mixture of serum with $\frac{N}{40}$ gives a deep blue. If we should be dealing with a blood whose alkalinity is appreciably diminished, of course all three mixtures would leave the colour of the test-paper unaltered. In such a case we should proceed to make a 50-, 60-, 70-, 80- and 90-fold dilution, and to make intermediate or still more extreme dilutions if required.

19. Method of expressing the results of the titration.

The most convenient expression for the alkalinity of the serum will be that obtained by specifying the dilution of normal alkaline solution which would, if it had taken the place of the serum in the titration, have given exactly the same colourations of the litmus paper.

SECTION V.—METHOD OF MEASURING THE ANTI-TRYPTIC POWER OF THE BLOOD FLUIDS.

20. Preliminary considerations.

(*a*) *Demonstration of the presence of tryptic ferment in leucocytes and of an anti-tryptic element in the serum.*—It may perhaps be well before embarking upon the question of the measurement of the anti-tryptic power of the blood fluids to satisfy ourselves of the presence of tryptic ferment in the leucocytes and of anti-trypsin in the serum. It has been customary to take for the demonstration of the tryptic ferment leucocytes derived from pus artifically produced by the injection of foreign substances, such as aleuron, or plasmon. It will

be preferable to work with leucocytes obtained direct from the circulating blood.

This can be done by two different procedures. Both depend upon the principle that the red blood corpuscles break down when brought into contact with water, while the leucocytes remain undissolved.

(1) We collect the blood from the finger in a simple capillary pipette, dilute it with five volumes of warm distilled water, and mix in the barrel of the pipette; set aside the hæmolysed blood to coagulate; then with a pipette direct a stream of water on to the clot and continue washing and removing the wash water until a contracted and almost colourless clot shall have been obtained.

(2) As before, we collect the blood from the finger in a capillary pipette. But we let it clot in the undiluted condition; and now, when coagulation is complete, we blow out the firm cylinder of clot into warm water, and let it remain there till all the hæmoglobin has dissolved (Plate II., Fig. 7, *d*). This second method has over the first an advantage of convenience.

In either case what we finally obtain is a colourless clot which, when examined under the microscope, is found to consist of a mass of leucocytes and detritus (stromata of red corpuscles and blood platelets) embedded in a meshwork of fibrin.

In each case the clot is now transferred to a tubule containing a small quantity of gelatine—the nutrient gelatine which is used as a culture medium will serve our purpose—and then, after it has been sealed up, the tubule is placed in an incubator or water-bath at a temperature of 50° to 55° C.—this being the optimum temperature at which to carry out a tryptic digestion.

In an actual experiment we should of course employ two control tubules. Into the first we should introduce gelatine without any other addition; into the second, gelatine, a washed clot, and a little serum.

Precisely similar clots would, of course, be employed. A convenient amount of blood to employ for each clot would be 20 cmm. Of gelatine 40 cmm. would be a suitable amount to fill into each tubule, and 5 to 10 cmm. would be a suitable amount of serum to add to the second control tubule.

After incubating the tubules for 2 to 3 days at 50° to 55° C.—this length of time would be required for the liberation of the tryptic ferment from the leucocytes—it would be found that the clot in the tubule without serum had dissolved by auto-digestion, while the clot in the tubule with serum had remained intact.

And on cooling the tubules in cold water it would be found that while the gelatine in the two control tubules had retained its power of

setting into a jelly, that in the tubule in which the clot had dissolved had been permanently liquefied.

(*b*) *Rôle played in the organism by the liberated tryptic ferment of the leucocyte and by the anti-tryptic power of the blood fluids; and pathological significance of variations in this power.*—It has only recently been realised that the tryptic ferment which is liberated from leucocytes and the anti-tryptic power of the blood fluids have important offices to perform in the organism. But already it may be taken as assured, on the one hand, that the liberation of tryptic ferment from disintegrating leucocytes accounts for the formation of the abscess sac in suppuration, for the burrowing of pus, and for the auto-digestion of the surface in the case of neglected wounds; and, on the other hand, that the anti-tryptic power of the blood fluids places a limit upon the operations of the tryptic ferment and preserves the tissues round an abscess sac, or other focus of inflammation, from progressive destructive digestion.

It is also assured that the anti-tryptic power of the blood is increased in suppurative processes and malignant disease to such an extent as to assume a considerable diagnostic importance.

Finally, it is not impossible—and the observations of my fellow-worker, Dr. Beaton, in many instances furnish support to this view—that a primitively deficient anti-tryptic power or a deficient anti-tryptic response may account for the rapid breaking down of a tubercular lung, or of tubercular glands, as well as for that rare idiosyncrasy which is characterised by the formation of aseptic abscesses at the seat of any hypodermic inoculation, and by indefinitely delayed healing and an exaggerated auto-digestion of any surface wound. Similarly a primitively excessive anti-tryptic power, or an exaggerated anti-tryptic response may perhaps account for those cases where we have continued induration instead of the formation of an abscess sac; and for the conditions in which we have pus which will not run, false membranes which will not come away, and sloughs which will not separate.

It is with a view to the study of this whole class of problems that the measurement of the anti-tryptic power of the blood assumes importance.

21. Consideration of the methods which are available for the measurement of the anti-tryptic power of the blood fluids.

Two different procedures suggest themselves by which the anti-tryptic power of the blood might be measured.

(*a*) We might, taking coagulated albumen, active pancreatic extract and serum, see in what proportions the serum and tryptic ferment

would neutralise each other so as to leave the coagulated albumen undigested.

(*b*) Or again—basing ourselves on Mellanby's work—we might take, instead of coagulated albumen, milk which had received an addition of calcium chloride, and again active pancreatic extract and serum, and see in what proportions the serum and rennin ferment would neutralise each other so as to leave the milk uncoagulated.

The first procedure would furnish a direct, the second only an indirect method for measuring the anti-tryptic power of the blood.

On the other hand the first method would, as compared with the second, have certain disadvantages. It would involve a longer period of observation; for while the clotting or non-clotting of milk would be decided in ¼ to ½ hour, the digestion or non-digestion of coagulated albumen could not be finally adjudicated upon in less than four to six hours. Again, while the clotting or non-clotting of milk gives in every case a sharp end point, with the coagulated albumen uncertainty would in some cases inevitably come in in connexion with the decision as to whether there was or was not some trace of digestion.

In view of this balance of advantages it will be well here to describe both procedures, and it will be clear that inasmuch as the digestion or non-digestion of the coagulated albumen would be judged of by inspection, and the clotting or non-clotting of milk by blowing out the tubes, the proper apparatus to use in the former method is the throttled composite pipette, and in the latter method the many-stemmed pipette.

I am indebted to my colleagues, Drs. Douglas, Noon, and Beaton, for valuable help and suggestion in connexion with both methods.

22. Direct method of measuring the anti-tryptic power of the blood fluids.

Principle of the method.—Progressive dilutions of an active pancreatic extract are mixed with diluted serum derived from the patient who is to be tested, and with a finely floccular suspension of coagulated albumen.* Precisely similar mixtures are made with a control serum.

Each of these series of mixtures is filled into a throttled pipette to be afterwards incubated at 50°C.†

* The arrangement by which "falling quantities" of trypsin are mixed with "level quantities" of albumen suspension and serum, presents over the arrangement in which "level quantities" of trypsin and albumen suspension would be added to "falling quantities" of serum the advantage that the albuminous content of each mixture is the same throughout the series.

† The digestion is carried out at 50°C., not only because this is the optimum temperature for tryptic digestion, but also because it ensures the sterility of the procedures

When the appointed time limit has expired the tubes are inspected to ascertain how far the clarification, which serves as an indicator of digestion, has progressed in the two tubes.

The results are expressed in the form of a fraction:—

$$\frac{\text{anti-tryptic power of the patient's serum}}{\text{divided by anti-tryptic power of the control serum}};$$

and this is spoken of as the *anti-tryptic index.*

Requisites for carrying out the method.—An efficient solution of trypsin*; pipettes with a proximal throttle; 0·5 % sodium carbonate solution for diluting the serum; a homogeneous suspension of a very finely divided coagulated serum albumen; the serum which is to be tested and a control serum; and a water-bath or incubator regulated to maintain a temperature of 50° C.

A very fine and homogeneous suspension of coagulated serum albumen can be made from sheep's serum by diluting it 5-fold, adding one drop of 4% acetic acid to each cubic centimetre of serum employed, and then applying heat. The albuminous precipitate thus obtained is filtered off, washed on the filter, and then rubbed up in a mortar with 0·5% sodium carbonate. The thick suspension thus obtained is allowed to sediment, the supernatant milky fluid being employed for the purposes of the test.

Where sheep's serum is not at hand, a fairly satisfactory suspension can be obtained by diluting a little human serum 5-fold with physiological salt solution, adding a trace of acetic acid, and then heating it in a water-bath.

Working Instructions.—Having in a preliminary experiment standardised your trypsin solution by putting up progressive dilutions of it with an 8-fold diluted normal serum and the albuminous suspension—mixing together in each case one unit-volume of each of the three solutions—take in hand the serum you propose to test and a control serum.

Begin by making an 8-fold dilution of each, using the 0·5% sodium carbonate solution as a diluent. When these dilutions have been made they are to be filled into tubules. Then, employing a simple capillary pipette or a graduated pipette, or, where minute accuracy is required, a semi-automatic pipette, make the required series of dilutions of your tryptic solution, and arrange them, using one unit-volume of each, in the orm of a series of drops upon one or more slides.

* Satisfactory solutions of trypsin can be obtained from any chemist. That supplied by Messrs. Allen & Hanbury, under the name of Injectio Trypsin Co., for hypodermic injection, can be recommended.

Add to each of these drops one unit-volume of the albuminous suspension, and then the same quantity of the 8-fold dilution of the serum you propose to test.

After thoroughly mixing together the three components of each drop, draw up a sufficient specimen of each into the throttled pipette, separating off the volumes by air-bubbles.

Then seal up both ends of the capillary stem, using for the sealing of the throttle, if it is not accessible to the peep-flame, a little melted sealing-wax carried down on the end of a capillary tube.

Now, go through exactly the same operations with the 8-fold dilution of the control serum, employing the same dilutions of trypsin and the same suspension of albumen. Finally, immerse both tubes in the water-bath or insert them into a hot air-chamber, and read off the results after any convenient period, say after six hours have elapsed, noting in the case of each tube how far clarification has progressed.

23. Indirect method of measuring the anti-tryptic power of the blood fluids.

Principle of the Method.—Progressive dilutions of an active pancreatic extract are mixed with diluted serum, derived from the patient who is to be tested, and with milk which has received an addition of 1% calcium chloride. Precisely similar mixtures are made with a control serum.

Each of these series of mixtures is filled into a many-stemmed pipette to be afterwards incubated in a water-bath at 37°C.

When the appointed time limit has expired the tubes are to be blown out into water to see whether the milk has coagulated.

Requisites for carrying out the Method.—The requisites for carrying out the method are exactly the same as for Method No. 1, except that we here employ, in lieu of throttled pipettes, many-stemmed pipettes; and in lieu of the suspension of coagulated albumen, milk to which we have added 1-10th of its volume of a 10% solution of calcium chloride cryst.; and finally in the respect that we substitute for the 0·5% solution of sodium carbonate which was employed for diluting the serum, a 0·85% solution of sodium chloride.

Working Instructions.—The progressive dilutions of the pancreatic extract, the dilution of the serum, and the mixture of these with the indicator (here the prepared milk) are carried out exactly as in Method 1.

When the mixtures have been made these are drawn up by the same technique as that described in connexion with the determination of the coagulation time of the blood (*supra Sub-section* 8. *Working Instructions*) into the tubes of the many-stemmed pipette.

The pipette is then, as in connexion with the technique just referred to, floated upon a water-bath standing at 50°C. Finally, after the lapse of fifteen to twenty minutes, the ends of the tubes are separately broken off and they are blown out into water.

APPENDIX TO CHAPTER VII.

Method of measuring the salt-content of the urine, serum or any other fluid.

Preliminary Elucidation.—The method which is here in question was devised in order to seek more definite information with regard to the excretory function of the kidneys than is afforded by the specific gravity bulb and the cryoscope. While the former takes cognisance of the sum total of substances dissolved in the urine, in particular of the urea and dissolved salts, as well as of any sugar and albumen, and while the cryoscope takes cognisance of the urea and salts and sugar, if present, the method which is here to be described eliminates the urea from consideration and takes cognisance, in the case where sugar is absent, only of the salt-content of the urine. In view of the fact that it does this, and that we are already furnished with a simple clinical method for the separate estimation of urea, the way is made smooth for that separate evaluation of the water-excreting, salt-excreting, and urea-excreting capacity of the kidneys which would seem to be called for in connexion with every case of renal disease.

The only really searching method of conducting such an enquiry will be to press upon the kidney a large surplus of water, of salts, or of urea, and to see what capacity it has for dealing with each of these.

In the case where the water-excreting capacity is to be tested, a litre or more of water would be administered and food would be withheld for some hours; in the case where the salt-excreting capacity is to be tested a salt meal would be administered, and fluid would be temporarily withheld; in the case where the capacity for excreting urea is to be tested a full protein meal would be administered along with a minimum of fluid.

In health, the ingestion of a litre of water upon an empty stomach and bladder will generally be followed by the excretion of over a litre of urine within the next two hours, and the evacuated urine will contain only traces of salt (sometimes as little as 0.1%).

In Bright's disease the ingestion of such a quantity of water may induce only a very small excretion of urine, and œdema may supervene.

In health, a dry salt meal taken upon an empty bladder will be followed by the excretion of a urine which is rich in salts. The urine

may under such circumstances contain as much as 3% of salt (expressed in terms of NaCl).

In Bright's disease the ingestion of such a meal will not induce the excretion of a concentrated urine. The salt content will often continue below 0·3% (expressed in terms of NaCl) and œdema may supervene.

Principle of the method.—The fluid whose salt content is to be measured is progressively diluted until a dilution is reached which completely hæmolyses half its volume of blood.

A standard salt solution is then progressively diluted until here also a dilution is reached which hæmolyses half its volume of blood.

Having by this use of blood as an indicator found out what dilutions of the standard solution of salt and of the fluid under examination are comparable dilutions, it will be clear that a very simple calculation will give us the salt content which is required.

Requisites.—Throttled composite pipettes, and a rubber teat; a standard salt solution; distilled water; watch glasses and slides; a glass pricker, and a bandage.

Working instructions.—Using distilled water as the diluting fluid, make by the method described in *Chapter IV., Sub-section* 13, a series of geometrically progressive dilutions of the fluid to be tested. Measure off two unit-volumes of each of these dilutions, and arrange these two unit-volumes in a series of successive drops on one or more slides. Now make a prick in your finger, and aspirate from this into the throttled pipette as many unit-volumes of blood as there are drops on the slide, and then blow out a unit-volume of blood into each drop, mixing very carefully. When mixture is complete draw up into the throttled pipette a sufficient sample of each of the mixtures.

If the dilutions have now been chosen in such a manner as to give, on the one hand, mixtures which contain sufficient salt to keep the corpuscles intact, and on the other hand mixtures which lake the blood, this will be seen at a glance, for there will be at the one end of the tube mixtures which are opaque and bright scarlet in colour, and at the other mixtures which are transparent and of the colour of crimson lake. If this is not so, or if the gaps between the successive dilutions are unduly wide, take in hand another pipette and make further dilutions to supplement those already made, and add as before to two unit-volumes of each dilution in each case one unit-volume of blood.

Having completed the first series of operations, and having inscribed in each case the dilutions upon the barrel of the pipette, we proceed to make a progressive series of dilutions of our standard salt solution. This standard solution may be an aliquot dilution of a normal sodium chloride solution; or a 1 per cent. sodium chloride

solution; or, if this happens to be at hand, a 0·85% physiological salt solution.

In the case where a 1% salt solution is employed we make by the method described in *Chapter IV., Sub-section* 11, a 2-fold, a 3-fold, a 4-fold, a 5-fold, and a 6-fold dilution from this, adding afterwards any supplementary dilutions which may be required.

In the case where we employ a 0·85 % salt solution, we make by the method described in *Chapter IV., Sub-section* 12, from this $\frac{1}{17}$th dilution, and then from this (1 in 200 dilution) a 2 in 3, a 1 in 2, a 2 in 5, and a 1 in 3 dilution, adding again any supplementary dilutions which may be required.

Having made these dilutions we, as before, measure off two unit-volumes of each and arrange them in series on one or more slides, mix with them one unit-volume of blood, and then draw up one unit-volume, more or less, of each mixture into the throttled pipette.

Having labelled this pipette, we place it with its companion pipette in an incubator, laying them both on their side, and we now await the supervention of coagulation and the retraction of the clot.

Method of reading off and recording the results.—On inspecting the pipettes after coagulation has occurred and the clot has contracted it will be found (*a*) that in the mixtures which contain sufficient salt to prevent hæmolysis there is a solid clot and that the fluid which has separated out is water-clear; (*b*) that in the mixtures where the salt content is sufficient to prevent complete destruction of the red corpuscles, while admitting of some hæmolysis, there is a much less solid clot, and the fluid which has separated out is coloured with hæmoglobin, and (*c*) that in the mixtures which do not contain enough salt to prevent complete hæmolysis we have instead of a clot which is weighed down by load of red corpuscles a transparent crimson jelly in which a meshwork of fibrin is only with difficulty discernible.

All these different appearances are well shown in the *Frontispiece*, Fig. 1.

If we now note the degree to which the test fluid and the standard salt solution respectively have had to be diluted to obtain complete hæmolysis—we may speak of these as the hæmolysing dilutions—and set these down as the first and second terms in a simple proportion sum, using the salt content of our standard NaCl solution as the third term, we shall obviously arrive at the percentage of salts (expressed in terms of NaCl) in the fluid we are testing.

Example.—The standard solution of NaCl employed was a solution containing 1% of NaCl. Complete hæmolysis is obtained in a 10-fold dilution of this solution, and in a 32-fold dilution of the fluid under examination. The percentage of salts (expressed in terms of NaCl) in that fluid would therefore work out as follows:—

10 : 32 : : 1%: *Answer* 3·2%.

CHAPTER VIII.

MEASUREMENT OF THE AGGLUTINATING, BACTERICIDAL, AND BACTERIOLYTIC POWER OF THE BLOOD.

1. Introductory.

When blood-fluids are brought into contact with bacterial cultures, these may be affected in a variety of different ways.

The bacteria which were before separate may adhere together to form clumps (*agglutination effect*).

They may be killed without being dissolved (*bactericidal effect*).

They may be dissolved in the blood-fluids (*bacteriolytic effect*).

And they may be so altered as to be readily ingested by phagocytes (*opsonic effect*).

Inasmuch as each of these effects can be obtained independently of each other, it is convenient, though none of the active substances can be isolated and separately examined, to assume that we have in the blood-fluids *agglutinins*, *bactericidins* and *bacteriolysins* and *opsonins*.

It is, however, important to bear in mind that when we speak of a serum containing any of these, we mean no more than that the serum exerts an agglutinating, bactericidal, bacteriolytic, or, as the case may be, opsonic effect.

SECTION I.—METHOD OF MEASURING THE AGGLUTINATING POWER OF THE BLOOD.

2. Preliminary elucidation.

Nature of the phenomena we are dealing with in agglutination.—Agglutination (by which we mean the assembling of particles into aggregates) and sedimentation (by which we mean the rapid settlement of these aggregates) are phenomena which may come under observation in connexion with every fine suspension.

While we have here specially to study the agglutinating action which the blood-fluids may exert upon bacterial suspension, we must not lose sight of the fact that agglutination and sedimentation come under observation also in connexion with red blood-corpuscles (*vide Chapter V., Sub-section* 9), and consideration will show that these phenomena are also familiarly known to us in connexion with suspensions of inorganic particles.

It is agglutination which transforms impalpably fine precipitates which would pass through the pores of a filter into masses which are sufficiently large to be held back ; it is agglutination which accounts for the clarification which takes place upon the addition of alum to muddy drinking water ; and it is agglutination which accounts for the rapid settlement of silt in the sea, and for the rapid building up of sedimentary rocks.

Dissolved salts (electrolytes) are the active agents both here and in the case where bacteria are agglutinated. Speaking generally, salts of monovalent elements are less active than those of bivalent elements, and these again less active than those of trivalent elements.

Wherever in the case of bacterial suspensions agglutination is produced by the unassisted action of dissolved salts, this is spoken of as *spontaneous agglutination*.

Among microbes—some such as the plague bacillus, the tubercle bacillus, and under certain circumstances the micrococcus Melitensis—

are very prone to spontaneous agglutination. Others—such as the bacillus typhosus—are much less sensitive.

In the case where serum brings about a *specific agglutination*, it operates by rendering the microbe more sensitive to the action of the electrolyte. The sensitising element in the serum is known as the "agglutinin"; the element in the bacterial body with which it combines is known as the "agglutinable substance." This last is not prejudically affected by sterilisation of the bacterial culture at 60° C. or by an addition of formalin. Agglutinins are elaborated in the organism in response to inoculations and auto-inoculations of certain microbes. In many cases small quantities of these agglutinins are present also in the normal serum.

It follows that quantitative, as distinguished from qualitative, methods, are réquired in connexion with the investigation of the agglutinating power of the blood-fluids. And it is to be observed that only changes in the direction of an increase of agglutinating power can be registered by our methods.

3. Purport of the method.

The method may be employed (*a*) for identifying the bacterial infection from which the patient is suffering, (*b*) for identifying a microbe which has been obtained by culture from a patient, (*c*) for determining in the case where such cultivation has furnished one or more species of microbe whether we have in hand the microbe or microbes responsible for the infection, and (*d*) for measuring the amplitude of the immunising response which the patient is making to his infection. In the case of (*a*) we must have at disposal a standard culture, in (*b*) a standard serum, and in (*c*) we must have the blood of the patient taken at various intervals after spontaneous or artificially induced auto-inoculation.

In (*a*), (*b*), and (*c*) we must carry our dilutions beyond the limit up to which the normal serum gives a reaction, in (*d*) beyond the limit to which the patient's serum gives a reaction.

4. Principle of the method.

Progressively increasing dilutions of a serum are made and are mixed in each case with an equal volume of a bacterial suspension. These mixtures, together with a control (bacterial suspension mixed with an equal volume of the diluent) are stored in a throttled pipette. Naked eye inspection tells us in what dilution of the serum agglutination and sedimentation are occurring.

For the purposes of reading off the end reaction it is the sedimentation effect which is taken into account.

5. Requisites for the carrying out of the method.

A throttled composite pipette furnished with a tightly fitting rubber teat; a bacterial suspension which is sufficiently dense to show as an opalescent fluid when viewed in a capillary tube; the serum of the patient and in some cases a control serum; physiological salt solution, or, as the case may be, a more dilute salt solution; watch-glasses and slides.

Particulars with respect to certain of these requirements.

Bacterial suspension.—In the case of cultures of the typhoid bacillus, the cholera vibrio, and the micrococcus Melitensis, which readily break up, forming a homogeneous suspension, all that is necessary is to pour physiological salt solution over the surface of a young agar culture; to stir this up in the fluid with a platinum needle until we have a moderately thick suspension; and then to allow any unresolved bacterial clumps to sink to the bottom. The supernatant fluid may then be aspirated into a simple capillary pipette, and, after it has passed up into the barrel and has left the capillary stem clear, the neck of the tube may be drawn out in the flame of a by-pass to form a conical-ended tubule (*Chapter III., Subsection* 14, *a*).

In the case of a culture of the plague bacillus or any other microbe which agglutinates "spontaneously" in physiological salt solution the same procedure may be carried out with a 1 in 1,000 salt solution.

In the case of a tubercle culture the felted growth which there presents itself can be resolved into its elements by prolonged trituration in 1 in 1,000 salt solution, and any residue of unresolved clumps may be removed by centrifugalisation. But it is much less laborious to employ the tubercle powder of Koch which is obtained by reducing the desiccated tubercle cultures to a powder of impalpable fineness by appropriate machinery. The tubercle powder here in question may be made up into a suitable suspension by rubbing it down in a mortar with 1 in 1,000 salt solution.

Tubercle bacilli which have been heated to 100°C. are no longer acted upon by agglutinins.

Where we have to deal with highly dangerous microbes, or where we desire to operate from day to day with one and the same bacterial suspension, we may employ in lieu of suspension of living bacteria either suspensions which have been heated for one hour to 60°C., or suspensions in salt solution to which 1% of formalin has been added.

Serum of patient, control serum, and salt solution for the dilution of these.—Ample and more than ample serum for our purposes will be furnished from the quantity of blood which is filled into an ordinary

blood-capsule. A control serum is required where we are not sure of our culture, or where from any other reason it may be desirable to check our results. Ordinarily we may employ physiological salt solution as a diluent for the serum. Where, however, we are dealing with microbes like the plague or the tubercle bacillus, a 1 in 1,000 solution of salt is to be employed instead.

6. Working instructions.

Having got together all the requisites, take in hand the throttled pipette and make a series of geometrically progressive dilutions of the serum by the method described in *Chapter IV., Sub-section* 13, taking the precautions set forth in *Chapter IV., Sub-section* 7, and arranging the dilutions in the form of a series of drops on a slide; and then place on the slide an exactly similar drop of salt solution to serve as a control. Now after first thoroughly washing out the pipette, taking care as you do so to avoid aspirating any moisture into the throttle, fill in, separating off with air-bubbles, as many volumes of the bacterial suspension as you have drops on the slide, and now blow out one volume of the bacterial suspension into each drop beginning with the control. (We have now in each case doubled the original dilution of our serum and converted our 2-fold dilution into a 4-fold dilution, and our 4-fold into an 8-fold, and so on.) This done, wash out the pipette again—taking the same precautions as before—and then, commencing with the control drop, draw into the pipette of each as much as is required to furnish a satisfactory sample, separating off, of course, with bubbles of air. Finally, seal the orifice of the capillary stem. Seal up also, where required, the throttle by carrying down into it a little melted sealing-wax on the end of a capillary tube. It will be well to do this where you are dealing with a living culture of a dangerous microbe, such as the micrococcus Melitensis, and also where you fear that the movements caused by the alternate expansion and contraction of the bubbles in the stem of the pipette may blur the results. Then set the pipette upright to sediment.

Where circumstances demand the employment of a control serum the whole series of operations will of course be gone through again with this.

7. Method of reading off and recording the result.

The progress of events may be followed by naked eye inspection, or it may be more minutely watched by the aid of a hand-lens. The occurrence of agglutination is signalised first by the bacterial suspension assuming a granular aspect (*Frontispiece*, Fig. 4, A). This becomes

more and more marked until the clumps become separately visible. Where the agglutination reaction is complete, and where a bacteriolytic action does not interfere with this, the bacterial clumps now rapidly settle down, leaving a perfectly water-clear fluid (*Frontispiece*, Fig. 4, B).

According to the nature of the bacterial culture and the potency of the serum these effects manifest themselves after a shorter or longer interval. In the case where the undiluted serum of a patient who is making satisfactory response to typhoid infection is mixed in equal volume with a suspension of the bacillus typhosus the reaction may manifest itself instantaneously. We may, in fact, thus make a "lightning diagnosis." In the 4-, 8-, 16- and 32-fold dilutions the effect may be expected to show up clearly in the course of a quarter to half an hour.

In the case of the micrococcus Melitensis the reaction develops much more slowly, although in connexion with Malta fever agglutination may be obtained in much higher dilutions than in typhoid fever—in exceptional cases in dilutions as high as 1 in 6,000.

When the end point of the reaction is to be read off we must institute an arbitrary time limit; a limit of 24 hours will be found convenient. After the lapse of that time we read off the highest dilution in which we have a complete, or, if we prefer to make this our criterion, an incomplete sedimentation reaction. In reading off our results we keep carefully in view all the possibilities of fallacy which are explained below.

8. Fallacies which have to be guarded against in reading off results.

The fallacies against which we have to be on our guard in reading off our results may be classified as follows:—

1. *Fallacy of settlement by gravitation.*—When we are reading off the sedimentation effect, we have, of course, to discriminate very carefully between the settlement which is referable to agglutination, and the settlement which occurs in every bacterial suspension under the influence of simple gravitation.

Where the pipette is kept under continuous observation we can readily discriminate the two kinds of settlement by the fact that the sedimentation which is referable to agglutination takes place comparatively rapidly, while the other is a very slow process.

In the case where the pipette has been put by and is examined for the first time after the lapse of a day or more, we can, however, still make the discrimination by attending to the following points:—

(*a*) In the case of a simple gravitation effect, the clarification will in the case of each separate test-mixture progress from above

downwards, and there will only be a very narrow band of perfectly clear supernatant fluid.

In the case of a settlement which is due to agglutination there will not be any such gradual tailing off of the opacity.

(*b*) In the case of a simple gravitation effect the sediment will take the form of an absolutely level deposit, for here the deposit, composed as it is of isolated bacteria, will be made up of impalpably fine elements.

In the case where sedimentation is due to agglutination, the deposit will be irregular and flocculated, and its general conformation will often recall to mind a heaped-up cumulus cloud. This appearance is, of course, due to the fact that the elements of which this deposit is made up are not isolated bacteria but clumps of bacteria.

(*c*) In the case where the settlement is due to gravitation alone, we shall, on inverting the pipette, see the deposit resolve itself into a shower of impalpably fine particles which will diffuse through the fluid and render it uniformly opaque.

In the case where the settlement is due to agglutination, the shower will consist of agglomerated masses of microbes which will rapidly sink to the bottom.

It will be appreciated that after a certain lapse of time the original agglutination effect will, in the case where it has not entirely clarified the fluid, be masked by a later supervening gravitation effect. The floccular deposit which testifies to agglutination will, in such a case, slowly be snowed under.

All the more important of these points are well shown in the *Frontispiece*, Fig. 4, A, B and C, and are more fully explained in the description.

2. *Fallacy of spontaneous agglutination.*—In dealing with microbes such as the tubercle and plague bacillus, which are very readily agglutinated by the action of salts, the fallacy of spontaneous agglutination can be avoided by employing both for the suspension of the bacteria and the dilution of the serum a 1 in 1,000 salt solution in lieu of the ordinary physiological salt solution.

3. *Fallacies arising from increased or diminished sensibility of the culture to agglutination.*—It has been observed in connexion with the micrococcus Melitensis, that cultures, which have been kept for a long time and which have presumably been sub-cultured only at long intervals, may be so modified as to agglutinate with normal sera in comparatively high dilutions. Serious errors have crept in in this way.

In connexion with the typhoid bacillus, Bordet's whooping-cough bacillus, and Pfeiffer's influenza bacillus, modifications in the contrary direction have been observed. Nothing has been elicited as to the reason of this, but the diminished sensitiveness to agglutination has, in the

case of the typhoid bacillus, been brought into relation with long continued daily sub-cultivation; and in the case of Bordet's bacillus, with cultivation on media containing too little blood. A very enigmatical series of changes, first in the direction of diminished sensibility to agglutination, and afterwards in the reverse direction, have been described to occur in typhoid cultures on heating these to temperatures above 60° C.

We can protect ourselves against the fallacies which are associated with increased or diminished sensibility of the bacterial culture, by putting up controls of normal bloods, or, as the case may be, of bloods of verified agglutinating power.

4. *Fallacy arising from the presence of bacteriolysins in the serum.*— Where we mix a serum with a bacterial suspension, with a view to measuring its agglutinating power, we must be prepared to find that the blood contains also bacteriotropic elements other than agglutinins. And where we are dealing with the typhoid bacillus and the cholera vibrio, we cannot afford to lose sight of the fact that the serum contains bacteriolysins. If this is not borne in mind we may, in the case where the serum is digested only in comparatively low dilutions and in the warmth with the bacterial suspension, fail to find evidence of agglutination, and erroneously infer that the serum contains no agglutinins. In the case where we have an extensive series of geometrically progressive dilutions such error is excluded. But it will constantly be observed—and not alone in connection with the typhoid bacillus and the cholera vibrio—that where agglutination is obtained, it is not by any means so clear and distinct in the lower dilutions of the serum as it is in the higher.

9. Points which have to be taken into consideration in comparing the data obtained in different estimations and by different methods of measurement.

Reflection will show that measurements of agglutinating power can be compared only where exactly comparable bacterial suspensions have been employed, and where one and the same criterion of agglutination is employed.

The first of these points does not stand in need of elucidation, and it will be clear that in making a sequence of measurements which shall be comparable *inter se*, we must employ throughout one and the same bacterial suspension, and by preference a formalinised suspension.

The second point is less clearly appreciated. A bacteriological worker who conducts his agglutination experiments in hanging drops, reading off his results with the microscope, and who attaches diagnostic importance, let us say, only to results obtained

in a dilution of 50 or 100 will, for instance, be outraged that a worker who employs the macroscopic method should attach diagnostic importance to a reaction obtained instantaneously in a 2-, 4-, 8-, or 16-fold dilution. But the standard of the two workers may, in reality, be practically identical. The microscopic worker is exacting in the matter of the dilution, but he is correspondingly more indulgent in the matter of the time limit, and he accepts as his criterion of agglutination the formation of the very smallest clumps. The macroscopic worker, on the other hand, relaxes his requirements in the matter of the limit of dilution, but he is correspondingly more exacting in the matter of the time limit, and accepts as evidence of agglutination nothing less than the formation of macroscopically visible clumps and the clarification of the supernatant fluid.

SECTION II.—METHOD OF MEASURING THE BACTERICIDAL POWER OF THE BLOOD.

10. Preliminary elucidation.

In the procedure employed by Koch—and after him by practically all bacteriologists—for the measurement of the bactericidal power of the blood, a bacterial suspension is added to the serum, and the bacterial population is enumerated by taking a loopful immediately afterwards, and other loopfuls at a series of appropriate intervals, and counting the number of microbes in these loopfuls by the method of plate cultivations.

The method is inaccurate in the respect that the unit-volume employed, the *loopful* is smaller or larger according as the loop, when withdrawn from the fluid medium, emerges from it edgewise, or disposed flat like a spoon when in use. And the customary method of measurement is also vitiated by two fallacies:—

(*a*) If the serum exerts any agglutinating effect the number of colonies obtained upon the plate stands in no relation at all to the actual population of microbes.

(*b*) Inasmuch as the reduction in the population which is effected by a bactericidal serum is afterwards masked by a multiplication of the survivors, the later counts—supposing these not to be further complicated by the fallacy of agglutination—represent not the actual reduction in the bacterial population, but the resultant of two quite unrelated phenomena, (*a*) the destructive force brought to bear by the serum upon the bacterial population, and (*b*) the activity with which the surviving population reproduces itself.

The method which is here described emancipates us both from the inaccuracy of the "loopful" and from these fallacies.

Principle of the method.—A series of volumes of serum is taken, and to these equal quantities of progressively increasing dilutions of an enumerated bacterial culture are added. The mixtures are maintained at blood heat, and after a suitable interval each is introduced into nutrient broth and cultivated to see whether a complete bactericidal effect has been exerted. The largest number of bacteria which the serum has been able to kill furnishes a measure of its bactericidal power.

11. Requisites for carrying out the measurement.

We require a specimen of blood aseptically collected in a blood-capsule (*Chapter V., Sub-section* 5); a young culture of the micro-organism upon which it is to be tested (a young culture is required because such a culture contains only a small proportion of dead microbes and the absorption of bactericidal elements by dead microbes is thus avoided)*; a supply of "looped pipettes" (*Chapter III., Sub-section* 4) one for each dilution of our culture; a graduated diluting pipette (*Chapter III., Sub-section* 9), and sterile water for washing this out; sterile nutrient broth for making our dilutions and cultivations; sterile tubules to store our dilutions and to serve as mixing tubes; a plasticine tray in which to arrange the tubules (Fig. 65, p. 175); and half-a-dozen sloped agar tubes for the enumeration of our culture. And where we propose to deal with the typhoid bacillus we may—as suggested by Capt. Smallman—conveniently employ for our test cultivations in lieu of ordinary nutrient broth a broth which has received an addition of mannite and litmus.

12. Working instructions.

It will facilitate the giving of detailed working instructions if we deal specifically with the measurement of the bactericidal effect exerted by the serum upon the typhoid bacillus, and if we divide up our whole procedure into a series of four operations.

Operation No. 1.—*Preparation of dilutions of the bacterial culture.*—Having made by the procedure described in *Chapter III., Sub-section* 14, *b*, a number of long rounded-ended capsules and having nicked them in the middle we break them across in an aseptic manner and arrange the rounded-ended tubules thus obtained in a row in the plasticine tray, sloping them as shown in Fig. 65, p. 175. We now wash out and carefully sterilise our diluting pipette and as soon as it has cooled down we fill in 250 cmm. of ordinary nutrient broth into each of the tubules.

Then withdraw from Tube 1, 5 cmm. of broth; from Tube 2, 2·5 cmm.; from Tube 3, 50 cmm.; Tube 4, 25 cmm.; Tube 5, 50 cmm.;

* Vide Chapter IX., Sub-section 8, d.

Tube 6, 25 cmm.; Tube 7, 50 cmm.; Tube 8, 25 cmm.; Tube 9, 50 cmm.; and from Tube 10, 25 cmm.

This done, transfer 5 cmm. of the typhoid culture to Tube 1 and 2·5 cmm. to Tube 2, making in this way a 50- and an 100-fold dilution.

Then bring the tube of sterile water to the boil and draw up the water into the diluting pipette in such a manner as to sterilise its interior.

After waiting a moment to allow the pipette to cool go back to Tubes 2 and 1 (in this order), and in each case draw up their contents into the pipette and blow them out again so as to effect proper mixture.

Next transfer from Tube 2 to Tube 3, 50 cmm. and to Tube 4, 25 cmm. of the 50-fold dilution, making a 500- and a 1,000-fold dilution.

Then re-sterilise the pipette with boiling water and go through the same series of operations, stirring up the contents of Tubes 4 and 3, and carrying on 50 cmm. and 25 cmm. of the 1,000-fold dilution from Tube 4 to Tubes 5 and 6, making a 5,000-fold and a 10,000-fold dilution.

Following on exactly as before make from the 10,000-fold dilution, a 50,000- and a 100,000-fold dilution ; and finally from the 100,000-fold dilution, a 500,000- and a 1,000,000-fold dilution.

Operation No. 2.—*Enumeration of the (living) bacteria contained in the bacterial culture employed.*—A 24-hour old broth culture of a vigorous strain of the bacillus typhosus will generally contain between 500 and 1,500 million living bacilli per cc.; and the million-fold dilution of such a culture will therefore contain 500 to 1,500 bacilli per cc.; and 10 cmm., *i.e.*, 1-100th cc. of such a dilution will contain 5 to 15 bacilli. This last will obviously be a very manageable number, *i.e.*, a number which can be spaced out comparatively widely on the area of a sloped agar tube; and a number which can, when each bacillus has grown out into a colony, be very easily enumerated.

We have thus at hand all that we require for the enumeration of our culture. We have at disposal both a 1-1,000,000-fold dilution and we can by the help of our graduated pipette, readily measure off and spread over the surface of agar 10 cmm. of the dilution.

All that it will be necessary to do will be to re-sterilise our diluting pipette by drawing up boiling sterile distilled water into it; then, after it has cooled down, to fill 10 cmm. of the 1,000,000-fold dilution; and then to spread this out very carefully over the surface of a sloped agar tube. We shall do well, with a view to controlling our count effectively, to plant out in this manner three 10 cmm. volumes on three separate agar tubes. If the culture we are employing is not very vigorous, and has not been sufficiently tested, it may be well to plant out, in addition, on three more agar tubes in each case, 5 cmm. of the 1 in 100,000-fold

dilution. But it will be well to note that it is always the thinnest sowing of bacteria which gives the most trustworthy count.

Operation No. 3.—*Filling in the series of looped pipettes with nutrient broth for the final cultivation test; mixing serum with the successive dilutions of the culture; and storing these mixtures each in a separate pipette, cut off from contact with the broth by a column of air.*

The method of procedure is as follows:—Taking a looped pipette, we place a mark upon its capillary stem with a glass-writing pencil at any convenient point—say at 1 to 1·5 cm. from the lower end, and fit a rubber teat to the barrel. The point of the capillary stem is now broken off between finger and thumb, the lower portion is sterilised in the flame, and the air is expelled from the teat.

Mannite broth is then aspirated into the pipette, until the bulb is about two-thirds full (Plate IV., *a*).

The end of the capillary stem is now inserted into the open end of the blood-capsule, which has been placed ready to hand. The serum is allowed to flow in until it reaches the pencil mark.

The orifice of the pipette is now raised above the surface of the serum, and a bubble of air is admitted into the tube to serve as an index for the next measurement.

This done, the end of the capillary stem is carried over into the tubule containing the particular dilution of the culture which is to be dealt with in this particular tube. (It will be convenient—inasmuch as we shall thus avoid the necessity of employing a separate mixing-tube for each dilution—to begin with the 1,000,000-fold dilution.) The culture is allowed to flow in until the bubble of air has just been carried past the pencil mark (Plate IV., *b*.)

The next procedure is to mix together the equal volumes of serum and culture which have been measured off. (Plate IV, *b*.) This is effected by blowing these two volumes out into a mixing tube and drawing up and driving out the fluid several times in succession. After a little practice this can be quite easily achieved without driving the sterile broth down from the bulb of the pipette into the lower part of the capillary stem and there contaminating it.

The column of mixed serum and culture is to be drawn up into the middle region of the capillary stem. It will be found that when the column is left in this position the intervening column of air which occupies the upper portion of the capillary tube will effectually isolate the fluid in the bulb of the pipette from the mixture of serum and culture. The lower orifice of the tube is now sealed; the teat is removed (Plate IV, *c*); the dilution which is in question is inscribed on the barrel of the pipette; and the filling of the series of tubes with the remaining dilutions of the culture is proceeded with.

When the whole series of tubes has been filled in, these are placed upright in a test tube labelled with the date and the source of the serum. The serum is then allowed to exert its influence on the bacteria with which it has been brought in contact for a fixed period. It is convenient to allow the serum to remain in contact with the culture for a period of 18 to 24 hours at 37° C.

Operation No. 4.—*Aspirating the mixtures of serum and culture into the reservoir of nutrient broth and incubating to ascertain whether a complete bactericidal effect has been produced.*

The steps of the procedure are as follows:—

The tubes having been taken in hand singly, the lower portion of the capillary stem is in each case heated in a peep-flame and drawn out into a throttle. (*Chapter II., Sub-section* 12, *a.*)

A condition of negative pressure is now established in the interior of the pipette by fitting over its upper end a collapsed rubber teat. While carefully regulating this negative pressure by keeping the finger and thumb in position on the teat, the finely-drawn-out end of the capillary stem is gently snapped across. The column of fluid will then be very quietly carried up into the bulb of the pipette. (Plate IV, *d.*) The end of the tube is then resealed.

When the whole series of pipettes has been dealt with they are returned to the incubator.

13. Method of reading off the result.

After the pipettes have remained in the incubator for another 24 hours the determination of the continued sterility or otherwise of the broth may be made by mere naked eye inspection.

Where a complete bactericidal effect has been exerted the nutrient fluid will of course not have undergone any colour change, and will have remained clear. (Plate IV, *e.*)

Where a growth of the typhoid bacillus has occurred there will be a perceptible cloudiness in the broth, and a very marked colour change, indicating that acid has been formed from the mannite, will have occurred. (Plate IV, *f.*)

If we had turbidity without a change of colour this would be indicative of the admission of contaminating microbes.

A simple numerical expression for the result is obtained by referring to the result of the enumeration (Operation No. 2) and calculating the number of bacteria contained in 1 cc. of the lowest dilution of the bacterial culture which has been completely sterilised. This will of course give the number of microbes which 1 cc. of serum would be capable of killing.

PLATE II

PLATE IV.

illustrates the method of measuring the bactericidal power of the blood. (*Chapter* VIII., *Section* II.).

a. Looped pipette which is being filled in with litmus mannite broth which will afterwards tell us whether the blood has exerted a bactericidal effect on the typhoid bacillus.

b. The nutrient fluid has been carried up into the cultivation chamber and one unit volume of serum and one unit volume of a dilution of the typhoid culture have been measured into the stem of the pipette.

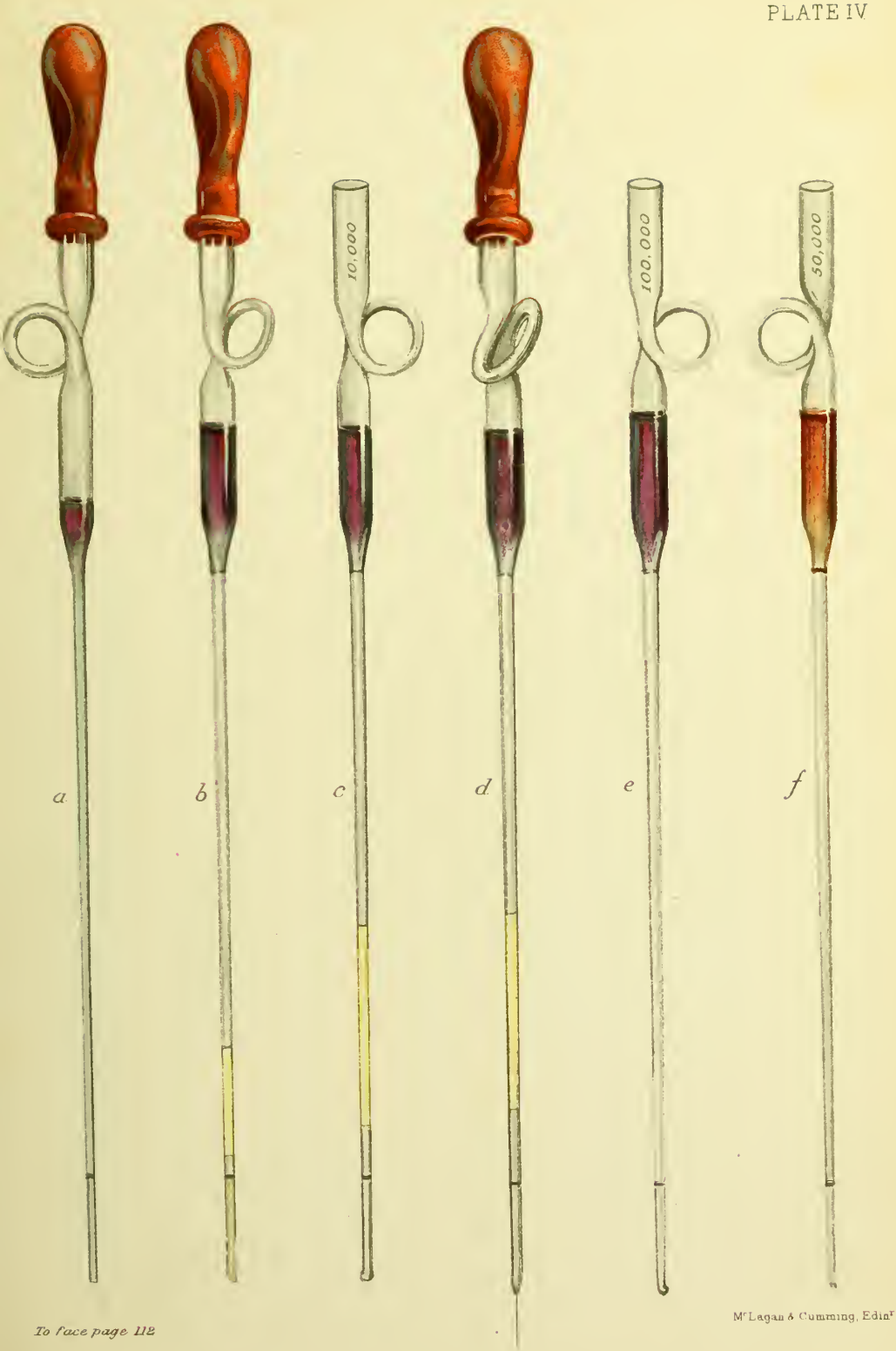
c. The serum and the dilution of the typhoid culture have been mixed and blown out, and have now been reaspirated into the stem, and the end of this has been sealed in the flame. The teat has been withdrawn and the dilution of the culture has been inscribed on the mouthpiece of the pipette.

d. After 24 hours in the incubator the test-mixture is being drawn up into the incubation chamber. To this end the capillary stem has been throttled, and the teat has been reimposed on the mouthpiece of the pipette in the collapsed condition.

e. The pipettes have been returned to the incubator for 24 hours in order to ascertain whether the typhoid bacilli have, or have not, been killed off by the action of the serum.

In the case of the particular dilution of the culture which was employed in this pipette they have been completely killed off. This is shown by the fact that the litmus mannite broth has remained clear and unchanged in colour.

f. In the case of the particular dilution of the culture which was employed in this pipette the typhoid bacilli have not all been killed off. This is evidenced by the change of colour and the slight turbidity and deposit in the nutrient medium. The change of colour is explained by the fact that typhoid bacilli produce acid when grown in litmus mannite broth.



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While a convenient basis for the comparison of a series of bloods is thus obtained, it must be understood that the expression just referred to is nothing more than an arbitrary formula expressing the bactericidal power of the serum when brought into application upon a particular culture in a 50 % dilution.

14. Special modifications and applications of the method.

The method described above may be modified to suit different requirements.

1.—If it is desired to determine whether any bactericidal effect is exerted by the practically undiluted serum, this can be done by making duplicate progressive dilutions of the culture, in the one case in serum and in the other case in nutrient broth, and cultivating from these.

2.—If it is desired to measure the bactericidal effect which is exerted by the serum in a practically undiluted condition, this can be done by making a progressive series of dilutions of the enumerated bacterial suspension in serum, and afterwards cultivating from these.

3.—If it is desired to measure the bactericidal effect of an antiseptic upon a culture, we make any convenient dilution of the culture, and a series of progressive dilutions of the antiseptic; and then follow the procedure described above, taking care to use in the looped pipette relatively large volumes of nutrient broth and minimal volumes of the mixture of antiseptic and culture.

4.—If it is desired to measure the bactericidal effect of the serum under anaerobic conditions, we modify the technique in the manner now to be described.

SECTION III.—METHOD OF MEASURING THE BACTERICIDAL EFFECT EXERTED BY THE SERUM UNDER ANAEROBIC CONDITIONS.

15. Purport of the method.

The method was devised in order to put to the test of experiment the statement of Metchnikoff that the blood does not exert a bactericidal effect under anaerobic conditions.

Experiment does not bear out this statement.*

16. Principle of the method.

Access of air is prevented by enveloping the blood, the dilutions of the culture, and the mixtures of serum and culture, in oil. Except in this respect, and in the respect that the nutrient broth may here advantageously be filled in at the end instead of at the beginning of

* *Wright.*—On the comparative bactericidal effect exerted by human blood drawn off and tested under aerobic and anaerobic conditions (Proceedings Royal Society, Vol. lxxi., 1902), and *Wright*, "Studies on Immunisation," pp. 72 and 73.

the procedure, the technique is the same as in the aerobic method described above.

17. Requisites.

In addition to the requisites which are set out above (*Sub-section* 11), we require a fatty-acid-free oil to serve as our enveloping medium for carrying out the method; a collecting thimble, made from a test tube, for collecting a specimen of blood under anaerobic conditions (Fig. 59, A), and a sterilised rubber cap for covering it in, such as employed for covering in test-tubes.

It is essential that the oil which is employed for the envelopment of the blood should be absolutely neutral; *first*, because the presence of fatty acid would diminish the alkalinity of the blood and precipitate its calcium salts, and might affect its bactericidal power; and, *secondly*, because an oil which contains fatty acids is emulsified when it is brought into contact with serum, nutrient broth, and alkaline fluids generally. Such an emulsification would interfere with that sharp separation of the oil from the enclosed fluids which is absolutely essential to the proper carrying out of the technique described below.

The procedure* adopted for the preparation of a fatty-acid-free oil is as follows:—

18. Preliminary work.

(*a*) *Method of making a fatty-acid-free oil.*—Take a reasonable quantity, say 300 cc. of a cheap variety of table oil (cotton oil?)† and introduce it into a litre flask along with 150 cc. of half saturated barium hydrate solution. Digest these together at 60° C. on a water-bath, and let the contents of the flask be well shaken up at intervals. After three hours, or less, the contents will have separated into three layers: an upper layer of more or less clear oil; a middle layer of barium soaps; and a lower layer of barium hydrate solution.

A drop of the supernatant oil is now tested by shaking it up in a test tube with some 0·25 per cent. sodium carbonate solution. If it rises to the top in separate globules, without any trace of emulsification, it may be taken to be free from fatty acids.

The contents of the flask may now be poured into a wet filter. The barium hydrate solution will come through the filter first. When it has quite ceased coming through a clean dry beaker is placed under the funnel, and the whole filter-stand is placed in a warm

* The procedure here described was that which was employed by the late Professor E. Külz in connexion with experiments on the fat-splitting power of the pancreatic juice.

† Unstable oils, such as olive oil, are unsuitable, inasmuch as these last are broken-up and converted into soaps when digested, as described above, with the barium hydrate solution.

chamber. By next morning the whole of the clear oil will have come through into the beaker, the barium soaps remaining behind on the filter.

If on shaking up the filtered oil in a test-tube with the sodium carbonate solution a trace of turbidity makes its appearance, and if the turbidity is increased by breathing into the test-tube and shaking-up again, this will point to an admixture of barium hydrate. Such admixture can be got rid of by shaking up the whole volume of oil with distilled water, and then leading through a stream of carbonic acid gas and refiltering. When the oil has been thus purified, it will be neutral to litmus paper. No milkiness will be developed when it is shaken-up with the sodium carbonate solution ; and the globules of oil will remain distinct and come up promptly to the surface.

The fatty acid-free oil thus obtained is introduced into a stoppered bottle and is kept sheltered from light.

(*b*) *Method of making a thimble for collecting blood under anaerobic conditions.* — Such a collecting-finger as is shown in Fig. 59, A, is very easily made by drawing out a stout-walled test-tube in the blow-pipe flame into a fairly wide stem and then sealing this off in the flame.

19. Working instructions.

1. *Procedure for filling the blood into the thimble without allowing it to come into contact with the air.*—Pour some oil, prepared as above, into a test tube and heat it to 140° C. in order to sterilise it. After it has cooled down, allow a drop to fall into a $\frac{1}{4}\%$ of sodium carbonate and shake it up to verify that it is still acid free, and then fill up the sterilised glass thimble with the oil nearly to the brim.

This done, sterilise the ulnar aspect of the little finger with flaming alcohol, and then puncture it in two or three adjacent points with a glass pricker.

A bandage is now wound round the digit, the tip of this last is immersed into the oil, and pressure is applied to the finger pulp. The blood as it emerges descends through the oil in the form of large globules.

When pressure on the pulp ceases to yield blood, the finger is momentarily removed from the oil, the bandage is loosened and re-applied, the finger is re-immersed into the oil, and pressure is again made on the finger pulp. When a sufficiency of blood has been collected, a sterilised rubber test-tube cap is drawn over the thimble. This last is then placed in a centrifuge, and the blood is, by a few turns of the machine, driven down to the lower narrow end of the tube.

After allowing an interval of ten minutes or more for coagulation—the blood, it may be noted, invariably coagulates under the oil*—centrifugalisation is resumed. The contents of the thimble will now arrange

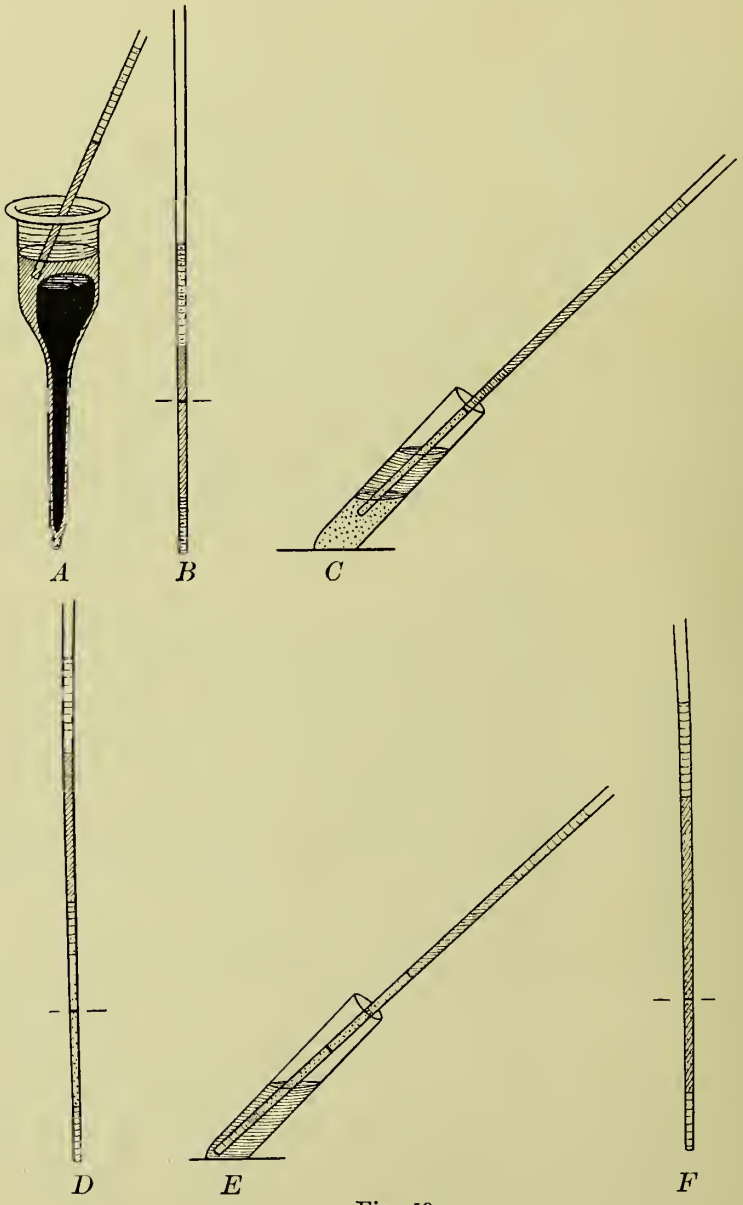


Fig. 59.

* The common text-book statement that coagulation is suspended when blood is collected under oil, is, it may be presumed, based on experiments undertaken with oil containing free fatty acids. It will be appreciated that these are decalcifying agents.

themselves into an upper layer of oil, a middle layer of clear serum, and a clot.

With a view to ensuring the asepticity of the further procedures, the serum may now with advantage be separated from the oil in the thimble, for this oil has been exposed to some risk of aerial contamination.

2. *Procedure for the separation of the serum from the oil in the thimble.* — The procedure is as follows: — A series of tubules which are to hold the serum and the dilution of the bacterial culture, are flamed, filled one-third full with sterilised oil, and then set up in a sloping position in a plasticine tray. A looped pipette fitted with a rubber teat is taken, and sterile oil is aspirated into it until the capillary stem and the neck of the bulb has been filled in. The tip of the pipette is then thrust down through the oil in the thimble into the layer of serum, and this last is aspirated into the pipette (Fig. 59, A). The point of the pipette is now drawn back into the covering oil and a little of it is drawn up in order to provide a seal for the lower orifice. The pipette when thus charged is inserted into one of the tubules which have been prepared, is carried down till it touches the bottom, and the serum is driven out under cover of the oil.

With a view to separating it more completely from the oil which has been exposed to the risk of aerial contamination, the serum is carried across in the same way and in a new pipette from the first into a second tubule.

Procedure for preparing the dilutions of the bacterial culture and for measuring off and mixing equal volumes of these and of serum without contact with the air.—The dilutions of the bacterial culture (which may, or may not, have been grown anaerobically) are made with a diluting pipette in the same way as in the aerobic method, except in the respect that the nutrient broth which is used as the diluent has been boiled up to remove the contained air, and that the tubules which serve for the reception of these dilutions contain oil to serve as a covering.

The serum and bacterial dilution are mixed by filling into a looped pipette *first* a sufficiency of sterile oil, *secondly* serum up to the mark on the stem, and *thirdly* another small quantum of oil (Fig. 59, B). Employing this last as a seal for preventing contact with the air, the pipette is carried across to the tubule, which contains the 1 in 1,000,000 dilution of the culture* (Fig. 59, C).

* It will be remembered that it has already been pointed out in connexion with the aerobic procedure—and the same applies of course here—that if we use one and the same mixing tube for mixing serum with a series of bacterial dilutions, we must always begin with the highest dilution and follow on from higher to lower.

It is now filled in with this last up to the mark on the capillary stem, and is then again sealed up by drawing in some of the supernatant oil (Fig. 59, D). Mixture of the serum and culture can now be effected either in the pipette itself, or as was suggested to me by my late fellow-worker, Sir W. B. Leishman, in a mixing tubule under oil. In the former case the contents of the pipette are drawn up by cautiously relaxing the pressure on the teat until the walls of the capillary stem fall away sufficiently to liberate the index globule of oil. This having been got rid of, a series of up and down movements of the combined column of fluid will bring about the desired mixture. Where a mixing tube is employed—and this is the better method—the point of the pipette is carried down to its floor, and the serum and bacterial dilution are driven out under the covering seal of oil (Fig. 59, E). They are then intermixed by alternately aspirating them into and driving them out from the capillary stem, care being taken that the pipette shall never be emptied of oil. Lastly we re-aspirate the mixed fluids from the floor of the tubule, then draw in a sufficiency of oil to form an effective seal (Fig. 59, F), and then, having withdrawn the point of pipette from the oil, allow air to enter and occupy the lower third of the capillary stem. Finally, we seal the orifice of the tube in the flame, and having now our mixture of serum and bacterial dilution sealed at both ends by oil, and safely housed in the capillary stem of our pipette, we follow on in the same way with the rest of pipettes and then place them all in the incubator for 24 hours.

Method of filling in the bulbs of the looped pipettes with sterile nutrient broth.

This is carried out in the following way:—

Sterile nutrient broth having been placed ready in a tubule or covered watch-glass, the capillary pipette which contains the highest dilution of the culture is taken in hand. A negative pressure having been established in its interior by fitting on a collapsed rubber teat, the lower portion of the stem is introduced into the flame of a peep-light in such a way as to heat it without allowing it to fuse and to collapse under the influence of the internal negative pressure. The sterilised extremity is now snapped off by plunging it while still hot into the nutrient broth. The inflow which takes place through the orifice thus provided is arrested by the pressure of the finger and thumb upon the teat as soon as the cultivation chamber is about two-thirds full.

The sealing up of the tube and the subsequent cultivations are carried out in exactly the same manner as in the case of the ordinary (aerobic) estimation.

SECTION IV.—METHOD OF MEASURING THE BACTERIOLYTIC POWER OF THE BLOOD.

20. Purport of the method.

The method was devised to demonstrate in connexion with typhoid vaccine the fact that the bacteriolytic effect which the normal human serum exerts upon a living typhoid culture, or upon a culture which has been sterilised at 60° C., is such as to make it quite superfluous previously to break up the bacteria in the vaccine by mechanical means.

The method serves also for measuring in a rapid manner the changes produced in the blood by an immunising response to an inoculation of typhoid vaccine, or a typhoid auto-inoculation.

21. Principle of the method.

Graduated dilutions of serum are incubated for half an hour at 37°C., with in each case an equal volume of a 24-hour broth cultivation of the typhoid bacillus or other microbe. The bacteriolytic effect is arrived at by examining under the microscope preparations made from the series of mixtures.

22. Requisites for carrying out the method.

Serum derived from the patient and a control serum (preferably a "pooled serum"); a 24-hour old culture of the typhoid bacillus or any other microbe which may be in question; a capillary pipette graduated into six equal divisions, the magnitude of the divisions being adjusted to the amount of serum at disposal; two or more many-stemmed pipettes to receive the mixtures of serum and culture; a rubber teat, slides and watch glasses and carbol-thionin or any other convenient staining fluid for staining the preparations.

23. Working instructions.

Make by the technique described in *Chapter IV., Sub-section* 15, a $\frac{5}{6}$, $\frac{4}{6}$, $\frac{3}{6}$, $\frac{2}{6}$, and $\frac{1}{6}$ dilution of the patient's serum and arrange these dilutions—there will be two unit-volumes of each—in successive drops on a slide. Complete the series by adding at the one end of the scale two unit-volumes of salt solution, and at the other end of the scale two unit-volumes of undiluted serum.

Then, using the same pipette, add to each drop two unit-volumes of the broth culture, and go through the series, mixing carefully, beginning with the salt solution control and ending with the highest solution of the serum. This done, draw up the series of drops into the separate tubes of a many-stemmed pipette, and place this in an

incubator or water-bath at a temperature of 37° C.—taking note of the time.

Go through exactly the same procedures with the control serum, and in each case, after incubation has been continued for 30 minutes, take out the pipettes, break off the ends of the tubes, and blow out the contents on to one or more slides, and then taking specimens of each of the series of drops, examine them under the microscope both in the unstained and stained condition. Note in each case the dilution in which there is the first sensible bacteriolytic effect, and the dilution in which there is a complete effect; and compare the results in the patient's and the normal blood, arriving thus at a *bacteriolytic index.*

SECTION V.—METHOD OF MEASURING IN ONE OPERATION THE AGGLUTINATING AND THE GROWTH-INHIBITING EFFECT WHICH IS EXERTED BY SERUM ON THE TYPHOID BACILLUS.

24. Purport of the method.

The method provides a simple and unlaborious procedure for measuring immunising response to typhoid inoculation, or auto-inoculation.

25. Principle of the method.

Progressive dilutions of serum are mixed in each case with an equal volume of a 1 in 10,000 dilution* of a 24-hour old broth culture of the bacillus typhosus.

A control mixture consisting in equal parts of sterile nutrient broth and culture is added, and the whole series of mixture is incubated at 37° C. After 24 hours these are examined to see (*a*) up to what serum-dilution the bacterial growth is so completely inhibited as to leave the nutrient broth water-clear; (*b*) up to what serum-dilution culture takes place in the form of agglutinated masses, and (*c*) beyond what point we have an evenly turbid culture such as is seen in the control mixture.

26. Requisites for carrying out the method.

Serum derived from the patient which has been drawn in an aseptic manner; normal serum (preferably a pooled serum) also drawn off aseptically; a 24-hour broth culture of the typhoid bacillus; a diluting pipette and sterile nutrient broth for making the dilutions; sterilised tubules for holding the dilutions of culture and serum; throttled pipettes, and a rubber teat.

* This dilution will be quite water-clear when viewed in a capillary tube, and will, in the case where our typhoid culture contains 400 to 1,000 million microbes in a cubic centimetre, contain 200 to 500 microbes in the 5 cmm., more or less, which will be employed in each bacteriolytic mixture.

27. Working instructions.

Make with the diluting pipette by the technique described in *Chapter III., Sub-section* 10, first a hundred-fold and then from this a 10,000 fold dilution, using as receptacles for these dilutions tubules set up in a sloping position in plasticine.

Then take in hand the serum, and using here conical-ended tubules as receptacles, make in these by the technique described in *Chapter IV., Sub-section* 13, a geometrically progressive series of dilutions, beginning with a 2-fold dilution and going up to a 248 dilution or further, and complete the series with a unit-volume of nutrient broth to serve as a control.

Then add to each successive unit-volume of serum, one unit-volume of the 10,000 dilution of the bacterial culture, and mix. Then draw up a sample of each of these mixtures into a throttled pipette, and after sealing up the end of the tube and the throttle, place it in the incubator. Then go through precisely the same operations with the control serum.

Next day take out the pipettes and read off the results.

CHAPTER IX.

MEASUREMENT OF THE OPSONIC POWER OF THE BLOOD.

Preliminary elucidation— Purport of the method—Principle of the method—Conditions which must be complied with if the opsonic index is to furnish a satisfactory expression for the opsonic power of the blood—Requisites for carrying out the method—The patient's serum, and points to be considered in connexion with this—The control normal blood and points to be considered in connexion with this—Bacterial suspension, and points to be considered in connexion with this—Preparation of the bacterial suspension—Washed corpuscles, and points to be considered in connexion with them—Method of preparing washed leucocytes for opsonic estimations—Preliminary work—The making of the "trial trip"—Procedures which may be resorted to when no pyagocytosis is found in the opsonic films—Collection and preparation of our apparatus and materials, and arrangement of the working bench—The carrying out of the actual opsonic measurement—Operation No. 1: The making of the phagocytic mixtures and the incubation of these—Operation No. 2: Removal of the phagocytic mixture from the opsonic incubator, and making of the film-preparations—Operation No. 3: Fixing and staining of the film preparations—Operation No. 4: Enumeration of the opsonic film—Number of leucocytes to be counted—Technique of counting and noting down the figures—Method of working out the opsonic index—Code of morality which ought to come into application in counting opsonic films—Methods by which we may determine whether a conformity or difference which we have found between two bloods corresponds to a genuine agreement or difference between these, or is to be attributed to a working error—Question as to what inferences we shall be warranted in drawing from our opsonic measurements in the case where we have satisfied ourselves that our working error falls out of account.

APPENDIX.

A consideration of the contention of the mathematical statistician that he has authority to pronounce judgment upon the number of leucocytes which require to be counted in the opsonic film.

1. Preliminary elucidation.

Introductory.—Phagocytosis of bacteria—and we here occupy ourselves only with phagocytosis of bacteria—may be discriminated into "opsonic phagocytosis," which is obtained when phagocytes are brought into contact with bacteria upon which the blood fluids have exerted an action which prepares them for ingestion (opsōno=I

prepare for table, I render palatable) and "spontaneous phagocytosis," which is independent of such action, and depends on the spontaneous activity of the leucocytes.

It is with the separate measurement of the former variety of phagocytosis that we shall here be concerned.

Problem as to the nature of "opsonins"; and summary of what has been elicited with respect to the opsonic action of the serum.—Attention has already in the last chapter been directed to the fact that while we have experimental knowledge of agglutinating, bactericidal, bacteriolytic, and opsonic effects, the use of the terms *agglutinins*, *bactericidins*, *bacteriolysins*, and *opsonins* is justified only by considerations of verbal convenience. But it will not be superfluous to reiterate this here. For the ordinary biological worker does not perceive that he is going out of his depth when he refers the bacteriotropic activities of the blood fluids to definite active principles, and discusses whether the above-mentioned hypothetical entities are separate and independent entities, or identical.

Thus much in justification of leaving severely alone the discussion of what does not come into the category of knowledge—*i.e.*, the nature of the opsonins, and dealing here only with what does come into that category—*i.e.*, the facts which have been elicited with respect to the opsonic action of the serum.

That action is, as has already been said, exerted by the blood fluids upon the bacteria. It is, in the case of the normal blood, exerted only by the unheated blood fluids; the opsonic action of normal serum being abolished by a short exposure to a temperature of 55° to 60° C. It is abolished also when complement is deflected. In the case of serum derived from patients or animals which have undergone frequent inoculations or auto-inoculations, opsonic action is, as a rule, not abolished* by exposure to a temperature which inactivates ordinary sera.

The opsonic action which the blood fluids exert upon each species of microbe is specific in the sense that, under the influence of infection, or of appropriate inoculations of vaccines, the opsonic power with regard to the corresponding species of microbes may be increased or diminished, while the opsonic power with regard to all other species of microbes remains unaffected. Further, as has been shown by Bulloch and Western,† the *opsonins for tubercle*

* With regard to the best composition of the bacterial emulsion for the demonstration of the opsonic power in heated "immune bloods," *vide infra*, Sub-section 8, *d*.

† Proceedings Royal Society, Series B., vol. lxxvii., 1906, reprinted in *Wright*, "Studies on Immunisation."

may be removed from the blood fluids without any of the *opsonins for staphylococcus* being withdrawn, and *vice versâ*.

2. Purport of the method.

Like all the other methods for the measurement of the bacteriotropic powers of the blood which have been described above, the method which is here in question aims (*a*) at diagnosing the presence of a bacterial infection; (*b*) at determining the gravity of that infection; (*c*) at supplying control and guidance in connexion with any therapeutic procedures, in particular any inoculations, which may be undertaken with a view to combating that infection; and (*d*) at determining whether an infection has been finally extinguished.

(*a*) When the method is employed *for diagnostic purposes* what we do is to search for evidence of auto-inoculation. That is to say, we seek to discover whether the natural bacteriotropic powers of the blood have undergone diminution under the influence of infection; or whether the patient's store of protective substances has been increased by the immunising response that infection may have called forth; or we seek for evidence of both the one and the other process, *i.e.*, of a negative and a positive phase occurring in sequence.

(*b*) When we wish *to determine the gravity of an infection* we in like manner seek to determine whether auto-inoculations are taking place; whether, if they are taking place, they occur *spontaneously*, or are elicited only under the influence of exertion; and finally, whether inoculation with a suitable dose of vaccine elicits satisfactory immunising response.

(*c*) When the measurement of the opsonic power is employed as *a guide in connexion with vaccine-therapy* our aim is to safeguard ourselves against diminishing the bacteriotropic power of the patient's blood; to certify ourselves that our efforts to increase that power have been successful; and to find out how long the store of protective substances that has been obtained for the patient remains in the blood.

(*d*) When we want to make sure, as a preliminary to leaving off treatment, that the *infection is extinguished*, we set ourselves to find out whether the expenditure of protective substances, which continues as long as infection subsists, is finally arrested.

3. Principle of the method.

An aliquot volume of the patient's serum, measured in a capillary pipette, is mixed with an equal volume of a bacterial suspension, and a like volume of a suspension of washed leucocytes derived from

a normal blood. After this "phagocytic mixture" has been digested for a suitable period at 37° C., film preparations are made and stained.

A "phagocytic count" is then undertaken, *i.e.*, the average bacterial ingest of the leucocytes in the phagocytic mixture is determined, and this is compared with the average ingest of the leucocytes in a phagocytic mixture made with normal blood.

The expression thus obtained,

$$\frac{\text{average ingest of the individual phagocyte in the mixture containing the patient's serum,}}{\text{average ingest of the individual phagocyte in the mixture containing the normal serum,}}$$

is denoted the opsonic index.

Two other methods of measuring and expressing the opsonic power of the blood have been proposed.

One of these—and it is a variant of the above—which is so obvious as to suggest itself inevitably to every mind—is to elicit, instead of the average number of microbes ingested by the individual phagocyte, the percentage of leucocytes which have ingested microbes. This method can give results only where the bacterial suspension has been standardised to give in the phagocytic mixture which is made with the control blood an appropriate proportion of blank cells; and it would seem that when the condition has been realised—and it may be difficult to realise—it gives results which do not go far apart from those of the standard method.

The *second* method—it is a method which has been employed with slight differences by Klien and by Neufeld—dispenses with the use of a control blood and measures the opsonic power of the blood by making a series of phagocytic mixtures with progressive dilutions of the serum in physiological salt solution and then counting the ingest of the phagocytes in the whole series of film preparations to see how far the dilution has to be carried to bring down the phagocytic count in the serum dilutions to that obtained with the simple unmixed physiological salt solution.

This method suffers, from the point of view of its application in actual practice, from the fatal objection that it involves the making of a quite indefinitely long series of progressive dilutions and the counting of a corresponding number of films.

It will be well here also to advert to the suggestion that it would be advisable to substitute for measurement of the opsonic power of the serum a measurement of the phagocytic power of the citrated blood—a measurement which would correspond very closely with the original method of my friend and late fellow-worker, Leishman, which furnished the point of departure for all quantitative work in connexion with phagocytosis.

I have elsewhere pointed out, in connexion with this suggestion, that an evaluation of the phagocytic power of the patient's blood in terms of the phagocytic power of the normal blood would not really bring us materially nearer to complete evaluation of the antibacterial powers of the patient. No thinking man can seriously entertain the belief that we have in the phagocytosis which takes place in our capillary tubes a full epitome of the complicated train of events which supervenes in the body when we have a microbic infection.

I have further pointed out that where we measure the phagocytic power of the patient's blood as a whole we are measuring the resultant of two different factors, and so learn nothing about the magnitude of either.

And again, where we have to deal with two factors, of which we can influence the one and not the other—and we can influence the opsonic power of the blood, but not the phagocytic capacity of the leucocyte—it will not be amiss, if we have any practical end in view, to confine ourselves to the evaluation of the particular factor which we can influence, or have a prospect of influencing.

4. Conditions which must be complied with, if the opsonic index is to furnish a satisfactory expression for the opsonic power of the blood.

The opsonic index will furnish a satisfactory measure of opsonic power; but it will do so only under certain conditions.

It is essential to its accuracy (*a*) that proper controls should be employed; (*b*) that a reasonable amount of phagocytosis should be obtained in the control serum; (*c*) that spontaneous phagocytosis should be suppressed in the phagocytic mixture; and (*d*) that the ingest of enough phagocytes should be counted.

(*a*) The requirement that suitable controls should be employed will be dealt with in its place.

(*b*) *Requirement that a reasonable amount of phagocytosis should be obtained in the control serum.*—There are certain species of pathogenetic microbes—such as the meningococcus, certain coliform microbes—and certain specially virulent strains of others—*e.g.*, of the pneumococcus—upon which the normal blood exerts either no opsonic effect or only a quite insignificant effect. In dealing with these microbes the denominator of our opsonic fraction will clearly be represented by zero, and in correlation with this, when in response to an infection by these microbes a certain amount of phagocytic power is developed in the patient's blood, phenomenally high indices will necessarily be obtained. These, while they will give us—as has been shown by Houston in connexion with meningococcic cerebro-spinal meningitis—quite invaluable aid in the diagnosis of the corresponding infections—will not betoken the acquisition of impressively high immunising power; nor will they be—inasmuch as they cannot be taken as quantitatively accurate—of much service in following out the progress of the disease.

(*c*) *Requirement that spontaneous phagocytosis should be suppressed in the phagocytic mixture.*—Where we set out to measure the opsonic action of the serum it will be a desideratum to suppress—so far as this may be possible—the spontaneous phagocytosis which may occur when leucocytes are brought in contact with most non-pathogenetic and pathogenetic bacteria, and in particular with the tubercle bacillus. The elimination of this factor is desirable, not only upon the general ground that the phenomenon which is being studied ought always to be uncomplicated by any other, but also on the ground that, when it is a question of spontaneous phagocytosis, enormous individual

differences may emerge as between leucocyte and leucocyte, with the result that the difficulty of arriving at a correct phagocytic count is seriously increased.

Our investigations—undertaken at the suggestion of my fellow-worker Capt. S. R. Douglas—have shown that spontaneous phagocytosis may be completely suppressed by bringing up the percentage of sodium chloride in the phagocytic mixture to 1% or over.*

(*d*) *Requirement that the ingest of an adequate number of phagocytes should be counted.*—This requirement will be discussed in connexion with the enumeration of the phagocytic film. The claim of the mathematical statistician that it rests with him to adjudicate upon this question receives attention in the Appendix at the end of this Chapter.

5. Requisites for carrying out the method.

The requisites for carrying out opsonic measurements may be grouped into two categories:—*apparatus*, and *materials employed in the test.*

The only special *apparatus* which is required is the opsonic incubator (*vide Chapter I., Sub-section* 8) and Hayden's ball and socket mortar (*vide Chapter I., Sub-section* 9). With the other apparatus, consisting as it does of simple capillary pipettes and their appurtenances, roughened slides, and the ordinary staining fluids, the reader is already familiar.

The *materials employed in the test* may be discussed under the headings—(1) *the patient's serum*, (2) *the control serum*, (3) *the bacterial suspension*, and (4) *the washed corpuscles.*

It will be convenient in connexion with each of these first to describe in general terms which is required; then to set out the procedure which is to be followed in its preparation; and, finally, to deal with its keeping properties.

6. The patient's serum, and points to be considered in connexion with this.

(*a*) *Programme which we may follow in taking specimens of the patient's blood for testing.*—In view of the fact that opsonic measurements may be required for the variety of different purposes which were enumerated above (*vide supra, Sub-section* 2), it will be well here to explain how to proceed in the matter of taking the specimens of blood required for these several purposes.

The procedures to be followed in connexion with the *diagnosis of the presence of infection* and the *determination of the gravity of the infection* will be very similar. We in each case take a series of

* *Wright*—"Studies on Immunisation," pp. 170, *et seq.*

specimens of the patient's blood drawn off at different times, and test them to see whether they furnish evidence of a fluctuating opsonic index.

We may in such a case—for the whole series of bloods will, of course, be tested together—dispense with the control of normal blood, and let the specimens of the *patient's bloods reciprocally control each other.*

Failing evidence of such spontaneous inoculations, we have recourse to one or other of the methods which are available for eliciting artificial auto-inoculations, and in connexion with this we take specimens of blood immediately before, half to one hour after, and again six hours after, and twenty-four hours after the procedure, which is designed to elicit an auto-inoculation.

When we have recourse to opsonic measurements for the purpose of *investigating the effects of an inoculation of a vaccine*, we draw off a specimen of blood before, and another twenty-four hours after inoculation in order to see, by a comparison of these specimens, whether we are obtaining an immediate positive phase or a negative phase. We take a further specimen of blood four to six days after inoculation, in order to certify ourselves that a production of protective substances has been achieved; and we again take another at the end of ten days to see whether the patient's store of protective substances is again running low. And, gradually, as the case progresses towards the extinction of the infection, and in correspondence with this the protective substances are less rapidly used up, we lengthen out this last interval.

Lastly, when, after tentatively giving vaccine-therapy up, we want *to see whether inoculations can be finally dispensed with*, we take a series of specimens of blood, leaving an interval of a month or more between the taking of the several specimens, with a view to seeing whether the opsonic power of the blood is maintaining itself without the help of inoculations.

(*b*) *Precautions which have to be taken in obtaining the serum.*—The patient's blood is filled into a blood-capsule in the ordinary way, and the serum is drawn off for use any time after the blood has coagulated.

Care must be taken to give sufficient time for coagulation to become complete, for if the serum is employed prematurely a secondary coagulation may occur when the phagocytic mixture is being incubated.

In drawing off the serum from the capsule care must be taken to avoid an admixture of corpuscles, for, as was shown by my fellow-worker, Dr. Fleming, such admixture entails—possibly because the

added corpuscles mechanically impede the leucocytes—a lowering of the opsonic index.

Serum which contains dissolved blood pigment in solution must always be looked upon with suspicion, for although the presence of a little dissolved blood pigment makes no difference to the index, and while it may be indicative only of the breaking up of a few red corpuscles by mechanical violence, or of the overheating of a minimal fraction of total blood, it ought always to suggest to the mind the possibility that an appreciable amount of the blood may have been overheated, and that opsonic power may by this means have been artificially lowered.

(*c*) *Keeping properties of the serum.*—While the question as to whether any appreciable changes occur in the opsonic power of the blood, in the first hour after the blood has been drawn off, is one which has not been definitely decided, it is certain that a condition of stable equilibrium with respect to the opsonic power is arrived at within a very short time after the blood has been withdrawn.

It is also certain that the opsonic power of all patients, except possibly those who are suffering from serious bacterial intoxication, remains—as does that of the normal serum—constant for two or more days; always assuming that the blood is kept in the dark, and that appreciable bacterial contamination has been avoided.

Blood for opsonic estimations may accordingly be sent to the laboratory through the post, but where it is a question of a lengthy journey, it will be well to take aseptic precautions in drawing off the blood and filling it into the capsule.

7. The control normal blood, and points to be considered in connexion with this.

All that has been said above with respect to the precautions to be taken in connexion with the patient's serum, and with regard to its keeping properties applies equally to the normal serum. Instead of dealing again with these issues we may therefore here pass to consider those which arise out of the fact that the normal blood, or a pool of these bloods (*Chapter V., Sub-section* 3) serves in connexion with the opsonic index as a standard of measurement; and that it serves also for controlling the accuracy of our work. We may consider these questions *seriatim*.

(*a*) *Question as to how far the individual normal blood or a pool of such bloods comes up to the requirements of a proper standard of measurement.*

The theoretical possibilities here would appear to be as follows:—

(*a*) *There may be no appreciable difference between one normal blood*

and another, from the point of the opsonic effect which it exerts upon pathogenetic bacteria.

If this obtains, the blood of every normal individual will furnish *an invariable standard of reference*, and at the same time a *standard of health* to which a patient's blood may be required to conform.

(*b*) *There may be great individual differences between one normal blood and another; but each such blood may represent a constant.*

If so the blood of one and the same normal man, and a pool of normal bloods will furnish an invariable standard of reference, but will not furnish a standard of health to which a patient's blood may be required to conform.

(*c*) *There may be great individual differences between the blood of one normal man and another, and there may, in the case of each, be important fluctuations in the opsonic power.* Here, as in the last case, a pool of normal bloods will be capable of furnishing an invariable standard of reference. And given that we know the limits within which the normal fluctuations are confined, and the rate of these, we may find it possible, in the case where we find a fluctuation in a patient's blood to say that we are dealing with phenomena which fall outside the range of the normal.

The above enumerated theoretical possibilities have not yet been adequately explored; but the following generalisations would appear to be justified.

(*a*) Where we are dealing with the tubercle bacillus, or any pathogenetic microbe which is foreign to the normal organism, or with the staphylococcus, or any other saprophyte of the external surfaces, which is normally shut off from all contact with the blood, the serum of one and the same normal individual, and *a fortiori*, the pooled serum of a number of normal persons, will furnish an invariable standard of reference. For the opsonic power of the blood does not—unless it be under certain quite exceptional circumstances—change with respect to the microbes with which we have no relations.

In accordance with this is the fact that there are only quite moderate differences—differences ranging perhaps from 0.8 to 1.2—between normal sera with respect to the opsonic power they exert upon the staphylococcus, and that in connexion with the tubercle bacillus the range of difference is even smaller, falling within the limit of the working error of a trained worker. In connexion with the gonococcus there appears to be a somewhat wider range.

It will thus be clear that there is in connexion with the tubercle bacillus and the staphylococcus (and in a looser sense in connexion with the gonococcus) and probably also in connexion with many other

varieties of microbes with regard to which systematic data are not yet available, a definite *standard of health.*

The question as to what diagnostic significance is to be assigned to a divergence from that standard is reserved for future consideration (*vide infra, Sub-section* 25).

(*b*) When, on the other hand, we are dealing with an intestinal microbe, or a saprophyte of the mucous membranes, the serum of a single person cannot be relied upon to furnish an invariable standard. For where intestinal microbes or saprophytes of mucous membranes are in question, every man will lie under the suspicion of having had relations with them. Here, therefore, it will be idle to look for a definite "standard of health," and it will not be legitimate to draw from the fact that a patient's opsonic power with respect to a particular microbe differs widely from the opsonic power of a pool of control sera, the conclusion that the disorder from which he is suffering is attributable to that microbe.

But that pool—supposing always that a sufficient number of normal bloods have been employed in making it—will furnish to us an invariable standard of comparison for the purpose of measuring the fluctuations which may occur in the patient's blood.

(*c*) *Question as to how normal bloods may be used to best advantage for the control of the accuracy of our work.*—Where it is a question of controlling the accuracy of our opsonic work, this can always be done by undertaking duplicate measurements and seeing how far these agree.

Where such measurements are undertaken, these may always with advantage be undertaken upon the control blood; for while the confirmation of the phagocytic count obtained with a particular patient's serum will corroborate only the numerator of the fraction which gives us the opsonic index of that particular serum, the confirmation of the phagocytic count obtained with the control blood will corroborate the denominator upon which depend the opsonic indices of the whole series of bloods which are under examination.

Furthermore, where we are dealing with a microbe in connexion with which there is a standard of opsonic power for the normal man, it will be well, where we have a choice between undertaking a duplicate or triplicate count upon a pool of control sera, and determining the opsonic power of the different control sera taken separately, to elect for this latter procedure. By following this procedure, we shall not only emancipate ourselves from any error of technique which might creep in in connexion with the collection of the individual sera or the pooling of these, but we shall be providing ourselves with an ideal series of cross checks, and shall at the same time be verifying that the

standard of measurement which we are employing is really the standard of health.

8. Bacterial suspension, and points to be considered in connexion with this.

(*a*) *Requirements in the matter of the morphology and staining properties of the microbes.*—The bacterial suspension which is employed must consist so far as possible only of elements which show typical morphological and staining characters. With view to this, the suspension must be made from a young and actively growing culture. In the case of most microbes a 24-hour culture will serve very well. But in the case of microbes, like the gonococcus and certain coliform bacilli, which rapidly undergo involution, it will be advisable to employ, when the rate of growth permits of this, cultures which are not more than 12 hours old. In connexion with the tubercle bacillus the best results will generally be obtained with cultures which are not more than 10 to 14 days old.

The following points also may be noted:

In the case of the influenza bacillus and its congener, Bordet's whooping cough bacillus, opsonic estimations are impracticable owing to the fact that there is no differential stain for these microbes, and that they do not with ordinary dyes stain more deeply than the protoplasm of the phagocyte.

In the case of certain other microbes—such as the glanders bacillus, and a variety of pseudo-diphtheria bacillus which is associated with the commonest and most obstinate form of uterine discharge—opsonic estimations are rendered difficult by the fact that the bacteria do not when stained with ordinary dyes stand out clearly in the interior of the leucocytes (see under *Staining of Opsonic Films, Sub-section* 19, *infra*).

In the case of microbes such as the gonococcus, and Friedländer's bacillus and certain other coliform bacilli which disintegrate rapidly in the interior of the cell, it may be necessary in the interests of clear staining to cut short the incubation time in the opsonic incubator (see under *Incubation of Opsonic Films, Sub-section* 18, *infra*).

In selecting a culture of the tubercle bacillus, it is well to choose one in which the bacilli are so far as possible free from beading, and one which contains a minimum of non-acid fast bacilli (ghosts). It will also be necessary when breaking up cultures with pestle and mortar to be on one's guard against breaking up the individual bacteria into fragments, or eviscerating them in such a manner as to leave only husks.

(*b*) *Requirements in the matter of the disintegration of the culture into isolated bacterial elements.*—Many varieties of microbes—and prominent among these are cultures of coliform microbes—give without any trouble quite uniform suspensions of isolated bacterial elements. Others do not fulfil these requirements. Staphylococcal cultures yield in addition to isolated elements clustered masses; streptococcal cultures cocci in chains, or sometimes only long chains; and the micrococcus catarrhalis very frequently only glutinous zooglœic masses; while cultures of actinomyces yield felted threads; cultures of diphtheroid bacilli felted bacterial masses; and tubercle cultures both felted bacterial masses and in some cases also zooglœic forms, which will appear under the microscope as collections of microbes spaced out in a faintly staining matrix (Plate V., Fig. 4.)

Accurate quantitative work is out of question if a bacterial suspension contains any considerable number of these aggregation forms, and in particular if it contains any appreciable number of felted or zooglœic bacterial masses.

It is therefore essential in the preparation of a suspension for use in an opsonic estimation to break up so far as is possible, or failing this to get out of the way, all aggregated masses of bacteria; and to prevent a reaggregation of the isolated bacterial elements in the bacterial suspension or in the phagocytic mixture.

The preparation of a bacterial suspension may accordingly involve our (*a*) triturating the culture or shaking up the suspension; (*b*) separating the residual clumps by centrifugalisation, (*c*) making choice of a suspending fluid which shall be inert, *i.e.*, incapable of inducing spontaneous agglutination, and (*d*) heating the culture to render the bacteria insusceptible to the action of the agglutinins of the serum. The detail of these procedures will be best described in connexion with the preparation of a suspension of tubercle bacilli where every one of these points must be attended to.

(*c*) *Requirements in the matter of the adjustment of the number of bacterial elements in the suspension.*—It is clear that it is a precondition of accurate and convenient working that the number of bacteria in the suspension shall have been so regulated as to give in the phagocytic mixture made with the normal blood an adequate number of bacteria in the average phagocyte without giving in any phagocyte too many to be correctly and easily counted. A tubercle suspension may advisably be regulated so as to give an average of 1·2 to 2·0 bacilli per cell, for if the intake of the average cell exceeds this there would be in a percentage of cells as many as six or more bacilli, and difficulties in counting would then arise owing to the fact that the bacilli would overlie each other and become

felted together. In the case where we are dealing with suspensions of cocci, or microbes like coliform bacilli, which have a perfectly well-defined shape and conform to a standard size, these difficulties do not arise, and so we may with advantage use a bacterial suspension which gives a larger intake.

The only absolutely trustworthy method of standardizing a bacterial suspension for opsonic purposes is the method of the *trial trip* (*vide infra*, *Sub-section* 13). Estimations of the opacity of the suspension, whether made with the naked eye or with any *nephelometer* may give fallacious results.

(*d*) *Requirements in the matter of sensitiveness to the presence of traces of opsonic power, and capacity for measuring differences in opsonic power.*—In connexion with these qualities in the bacterial suspension, the following facts, which were elicited by my fellow-workers, Capt. A. F. Hayden and Dr. W. Parry Morgan, would seem to be of quite fundamental importance.

An ordinary bacterial emulsion, and in particular such a tubercle emulsion as is obtained by the method described below, consists of two elements (*α*) intact bacteria, and (*β*) bacterial detritus.

These can be separated from each other by centrifugalisation and decantation, and the effect exerted by the detritus can then be studied by adding it to the washed bacilli in graduated quantities.

(*α*) When this is done and phagocytic mixtures are made the bacterial ingest is found to become smaller and smaller with each progressive addition of bacterial detritus. The result is easily explained. The detritus consists of free receptors which are chemically similar to the fixed receptors in the intact bacilli, and as more and more of the opsonin of the blood combines with the free receptors, little, and finally none, is left over to combine with the fixed receptors, and opsonise the intact bacteria.

(*β*) When the number of bacilli in the suspension is regulated in accordance with the instructions in (*c*) *supra*, when in other words the bacterial ingest is kept within the limits which allow of its being accurately enumerated, the following further effects manifest themselves.

In the absence of the bacterial detritus, every tuberculous patient's blood gives either a normal tuberculo-opsonic index or an index which approximates closely to the normal.

When a certain quantum of bacterial detritus is added every representative batch of tuberculous patients is found to furnish, in addition to bloods with a normal index, bloods with a sub-normal index.

When still further bacterial detritus is added we obtain in addition to normal and low indices also a series of high indices.

"These results—I quote here from the original paper of Hayden and Morgan*—admit of simple explanation. Reflection shows that when we are testing a series of sera with a view to eliciting differences in their agglutinating, bactericidal or opsonic powers, we cannot expect that the full differences will be revealed unless the bacterial suspension which we employ contains more microbes than the strongest serum is competent to agglutinate, kill or opsonise. And if the bacterial suspension contains only such number of microbes as the weakest serum is competent to agglutinate, kill or opsonise, no differences can be expected to emerge between the several sera. We cannot, for instance, in the case where we are dealing with a batch of sera of which the strongest is able to kill 6,000,000 typhoid bacilli, while the weakest is able to kill only 600,000, expect to elicit any differences in bactericidal power with a suspension which contains no more than 600,000 bacilli. Nor could we, if we employed a suspension containing only 3,000,000 bacilli hope to differentiate between sera that can kill 3,000,000 bacilli and sera which can kill up to 6,000,000.

"In the same way, when dealing with a batch of sera of which the strongest would be competent to opsonise sufficient microbes to give an average ingest of 10 bacilli per cell, and the weakest only enough to give an average ingest of 2.5 microbes per cell, we cannot expect to bring out any differences between the sera with a suspension which could provide at most 2.5 microbes per cell. Nor again, if we employ a suspension which could not provide more than 5 bacilli per cell could we hope to differentiate between sera which would be competent to opsonise sufficient microbes to give this count and sera which would be able to opsonise sufficient microbes to give us a count of 10 bacilli per cell.

"It might, in view of this reasoning, seem as if the only satisfactory suspension in the cases above considered would be: for the bactericidal test, a suspension containing six or more millions of typhoid bacilli; and for the opsonic test, a suspension which could provide for an average ingest of 10 microbes per cell.

"But this is not so.

"All that is required is that each suspension should contain a quantity of receptors equal to that which will be contained in suspensions of the required strengths: and Wright and Windsor† have shown in connexion with the bactericidal power of the blood that if sera are partially depleted of their bactericidal power by the addition

* Sent in for publication in the "Proceedings, Royal Society."

† "Journal of Hygiene," Vol. II., No. 4, Oct. 1902, and "Studies on Immunisation," page 45, *et seq.*

of receptors in the form of dead typhoid bacilli, quite weak suspensions of living typhoid bacilli will serve for the purposes of a differential test.

"The same thing holds in connexion with the opsonic power. If we partially deplete our sera of opsonic power by the addition of detritus to the phagocytic mixture, we can use quite weak suspensions of intact bacilli for the purposes of a differential opsonic test and still keep the bacterial ingest within the limits which will allow of its being accurately enumerated."

We have here what would seem to be a complete explanation of the fact that a bacterial suspension which contains no detritus and less intact microbes than the strongest serum would be capable of opsonising is a very unsatisfactory indicator of differences in opsonic power, while such a suspension becomes, when a full equivalent of bacterial detritus has been added, a satisfactory indicator.

The facts which have been brought out above and these considerations have a manifold importance. They tell us what we have to aim at in making a bacterial suspension (*vide infra*, *Sub-section* 9, *c*). They also make clear that two observers who are using different emulsions must often arrive at different opsonic indices. And they make it possible to understand how one observer, working with heated bloods derived respectively from a tuberculous patient and a normal man, finds phagocytosis in the first and not in the second; while a second worker using the same bloods finds no appreciable phagocytosis in either; and a third finds the same amount in both.*

9. Preparation of the bacterial suspension.

(*a*) Where we are dealing with a species of *micro-organism whose culture can be readily resolved into separate bacterial elements*, it is a very simple matter to prepare a satisfactory bacterial suspension. A tube, or a portion of a tube, of sterile physiological salt solution, is poured on to a young culture which has been grown upon an agar slope†; the microbial growth is carefully scraped off from the surface and thoroughly stirred up with a platinum needle; and this done the tube is set down for a few minutes to allow of any unresolved bacterial masses settling to the bottom. The upper layer of the suspension is now aspirated into the barrel of a simple capillary pipette. This after it has been converted into a tubule by burning through the

* It is clear that these results might be expected if the first worker was using a bacterial suspension which contained a sufficiency of detritus to differentiate between the two bloods; the second, a suspension which contained an excess of detritus; and the third, a suspension containing little or no detritus.

† If by misadventure particles of agar have been mixed up with the suspension, it is advisable to throw the whole of the fluid on to a small loosely woven filter and to employ the filtrate instead of the original suspension.

capillary tube just below the neck, furnishes a convenient receptacle, in which the suspension can, if necessary, be centrifugalised, and from which it can be drawn off as required.

(*b*) Where we are dealing with a *bacterial growth which does not break up spontaneously* into separate bacterial units the technique which has been described above must be supplemented. Where we are dealing with the staphylococcus it will generally be advisable to transfer a large loopful of the culture to an empty watch glass and then using this as a mortar and the moistened end of a centrifuge tube as a pestle to break up the culture by trituration, afterwards adding further salt solution drop by drop as required, and finally centrifuging to remove any clumps that may not have been broken up in this way.

(*c*) In the case where we have to deal with the *tubercle bacillus* the following more elaborate process of trituration is required.

Take a 7 to 10-day culture of the tubercle bacillus grown on glycerine broth; sterilise it by short heating in the steamer. Then collect the bacterial growth upon a filter, wash with sterile physiological salt solution and then place the filter paper in the incubator to dry. When dry the bacterial growth will separate from the filter paper and may then be transferred to a stoppered bottle, and may be kept indefinitely.

Take about 10mg. of this dry culture, place it in a ball and socket mortar, and reduce it to a powder by one or two turns of the pestle. A drop of 1% saline is now added with a pipette, and is rubbed into the powder until it is found that the pestle works evenly over the sticky paste. Holding now the handle of the pestle with the thumb and middle finger, and using slight pressure, triturate the paste for three to five minutes, occasionally lifting the pestle to scrape its edges with the blade of a penknife to bring the material which has collected on the rim of the mortar into the centre, and to add another drop of saline solution if the working becomes uneven from deficient moisture. The grinding completed, the pasty material in the mortar is stirred up into 1% saline solution, two or three drops being added at a time until about 20 drops have been added and a very thick emulsion has been obtained. This is transferred with the pipette to a "centrifuge tube," the pestle and mortar are washed with more saline solution, and these washings are added to the thick suspension until this has been brought up to the bulk of about 1cc.

The centrifuge tube is then placed in the centrifuge and is centrifugalised for about 1½ minutes in order to throw down the gross clumps. The supernatant suspension is then pipetted off into a clean centrifuge tube and it is further centrifuged to produce the working

suspension. The time required for this final centrifuging varies with the speed of the centrifuge and the character of the particular suspension. It may vary between 3 minutes and 20 minutes. The centrifugalisation completed, the supernatant suspension down to about 0·5cm. from the bottom of the tube, is pipetted off into a tubule, is diluted, if necessary, and then, seeing that it is impracticable to gauge the number of intact microbes correctly from the degree of opacity, a trial trip is undertaken, using for this purpose a phagocytic mixture consisting of a unit volume of the tubercle suspension mixed with a unit volume of normal serum and a unit volume of washed corpuscles.

The suspension may be adjudged to be satisfactory if in films made after incubating for the 15 minutes 120 to 200 tubercle bacilli are found in 100 leucocytes; if approximately the same numbers of bacilli are found in twin preparations respectively decolourised with 25% and 2·5% sulphuric acid; if there is a complete, or all but complete, absence of both bacterial clumps and zooglœic masses; and if both high and low indices are obtained in the case of a typical batch of bloods taken from tuberculous patients.

If these are not obtained the proper remedy will—as explained *supra*, *Sub-section* 8, *d*—be to add some of the supernatant fluid which is obtained by thoroughly centrifugalising a fairly dense tubercle suspension.

(*d*) Where we are dealing with *microbes which are dangerous to work with*, such as the bacillus pestis, the micrococcus Melitensis, or the glanders bacillus, we first kill the culture by pouring on a strong formalin solution (in the case of the glanders bacillus the 40% solution is not too strong), then stir up the culture in the formalin, transfer to a centrifuge tube, and centrifugalise until the bacteria have been carried to the bottom. Then pipette off the supernatant formalin, wash in physiological salt solution, pipette off again, after further centrifugalisation, and finally stir up in sufficient salt solution to make a satisfactory suspension.

Keeping properties of the bacterial suspension.—Once made the bacterial suspension will keep practically unchanged for many hours at the ordinary laboratory temperature. If it is placed in the ice chest the suspension may even be kept over from one day to another, or even from day to day for a succession of days. But where this is done it is clearly inevitable that there should be a certain settlement of the microbes and bacterial detritus. Further, in the course of time, even apart from the intrusion of contaminating microbes, degenerative changes will supervene in the bacteria. Elements of the bacterial body will no doubt pass into solution, and there will also be changes in the outer envelope of the bacteria which will lead

to the coalescence of the separate bacteria into zooglœic masses* (Plate V, Fig. 4).

In conformity with what has just been said, it will, when employing for opsonic work a bacterial suspension which has been kept over, be imperative to stir up the suspension in a thorough manner, and also to assure oneself that there is no bacterial contamination, and that no zooglœic masses have been formed. It will further, in the case where there has been any opportunity for admixture of serum with the suspension, be necessary to make sure that precautions have been taken against this.

10. Washed Corpuscles and points to be considered in connexion with them.

The washed leucocytes which are required for opsonic estimations are obtained by puncturing the finger, receiving the blood into a decalcifying solution, centrifugalising, decanting off the supernatant fluid and re-centrifugalising in salt solution until the corpuscles are adequately washed. By this method the leucocytes which we require are obtained in combination with red corpuscles.

The details of the procedure which is to be followed are described in a subsequent section, but it will be convenient here to direct attention to the following general requirements.

(*a*) The suspension of corpuscles which is employed in the phagocytic mixture may conveniently consist of corpuscles and fluid in approximately the same proportions as the normal blood.

(*b*) It is essential that the leucocytes should not have been agglomerated into groups in the process of centrifugalisation. They ought to be separate, and evenly distributed among the red corpuscles.

(*c*) With a view to the avoidance of anything of the nature of coagulation in the phagocytic mixture as well as to the avoidance of the fallacies which would be associated with the presence of opsonins in the suspending fluid the last traces of plasma ought to have been removed by washing.

(*d*) The fluid in which the corpuscles are suspended ought to be approximately isotonic with the blood fluids, and free from all bacterial contamination.

(*e*) The addition of a foreign serum ought not to agglutinate the corpuscles.

The necessity of avoiding any agglutination of red blood corpuscles in the phagocytic mixture was demonstrated by my fellow worker, Dr. A. Fleming. He showed that as soon as the red blood corpuscles

* It would seem that the formation of these zooglœic masses can be prevented or delayed by taking the precaution to render the suspension very faintly alkaline.

run together into clumps the count becomes irregular, and unduly high values are obtained, the former result being no doubt due to the irregularity introduced by the agglutination into the previously uniform mixture, and the latter result, it would seem, to the fact that in the large clearances between the groups of agglomerated corpuscles the leucocytes would have more unimpeded access to the microbes* than in the narrow interspaces between the separate corpuscles of the normal phagocytic mixture.

The requirement that agglutination of red blood corpuscles in the phagocytic mixture must be avoided can be complied with by drawing the blood which is to furnish the washed corpuscles from a person whose corpuscles have been found insensitive to the agglutinins which here come into consideration. Perhaps one out of every two normal men possesses such insensitive corpuscles, and this insensitiveness may extend even to the agglutinins which produce the characteristic agglutination in the blood of the septicæmic or gravely intoxicated patient.

Keeping properties of the washed corpuscles.—Washed corpuscles may be kept at the temperature of the room from morning to evening without deteriorating in any way. Far from deteriorating during this interval they show, when taken in hand at the end of the day, after standing in physiological salt solution, a distinctly increased phagocytic activity.

On the next morning, however—even if they have been kept in the ice-chest overnight—the leucocytes will be found to have lost a good deal of their original phagocytic activity, and they will be found to have become much more fragile. They will break up very easily, and give only very unsatisfactory films.

11. Method of preparing washed leucocytes for opsonic estimations.

Take a tubule—we may call it a corpuscle-tube—of the size and shape shown in Figure 60, and fill it about two-thirds full with a solution of 1·5% citrate of soda which has either been freshly made up, or has been kept sterile.†

Then draw blood from the finger in the way described in *Chapter V., Sub-section* 1, receiving it directly into the citrate of soda solution, and after every addition of blood invert the tube over your thumb in order to secure thorough mixture.

* It may be pointed out in this connexion that where by the continuous agitation of a phagocytic mixture provision is made for effective bringing together of the phagocytes and microbes, the phagocytic count may be increased five-fold and over.

† If attention is not given to the sterility of the solution we shall very often find our washed corpuscles contaminated with a growth of the bacillus mesentericus.

When sufficient blood has been collected—the amount required will of course depend on the number of opsonic estimations which are to be undertaken—place the tube in the centrifuge, carefully counterpoising by placing a similar tube containing water in the opposite bucket. Now set the centrifuge in motion.

It is very important to regulate carefully the amount of centrifugal force which we apply.

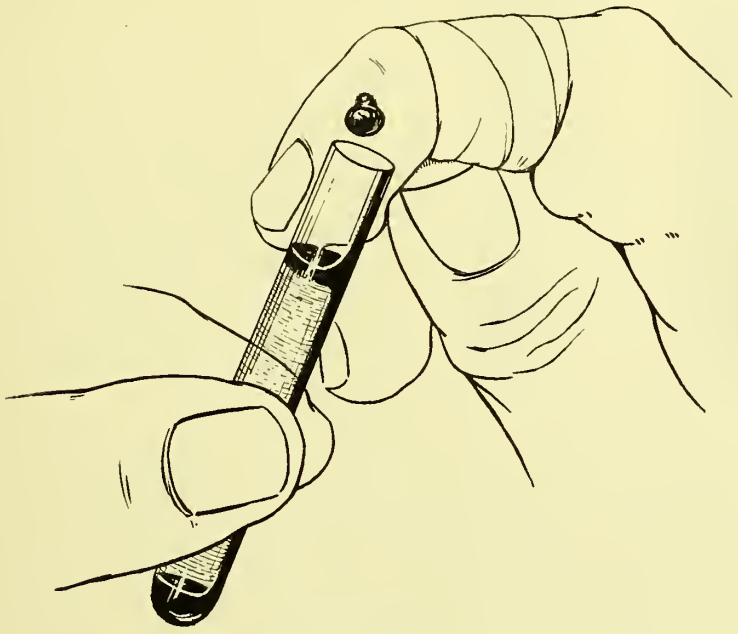


Fig. 60.

If we are to avoid losing a considerable percentage of our leucocytes and at the same time to pipette off a satisfactory amount of supernatant fluid, centrifugalisation must be continued until the upper limit of the deposit of corpuscles is sharply marked, and until we have a perfectly clear supernatant fluid.*

If we are to avoid packing our corpuscles too tightly and assembling our leucocytes into clusters, we must arrest the centrifuge as soon as the corpuscles have been compressed into about the same cubic space as would have been occupied by the volume of blood which was originally employed.

For the purpose of judging of this it is convenient to place a guide mark upon the corpuscle tube, as in Fig. 61, A and B, at the level at

* If any difficulty is found in carrying out this instruction, this will be due either to the inefficiency of the centrifuge, to want of attention in counterpoising, or to stopping the instrument too suddenly.

which the citrate stands before the blood is filled in. With this to guide the eye, we can quite well judge the space the centrifuged corpuscles ought to fill at the bottom of the tube.

Fig. 61.

After a few timing experiments the element of trial and error can be entirely eliminated, and it will be possible to fix the exact time required to bring down the corpuscles in a satisfactory manner.

When this time has been arrived at it will be well to adhere rigorously to it, for where one is dealing with a powerful centrifuge it will be found even so small an excess as 20 seconds over the optimum time limit will bring the leucocytes together into clumps.

As soon as the corpuscles have come to the bottom in a satisfactory manner, pipette or siphon off the supernatant fluid nearly down to the level of the corpuscles.

The tube is then filled up with sterile 1% salt solution, and several times inverted so as to wash the corpuscles, and it is then recentrifuged. But as the corpuscles are now suspended in a fluid which is less viscid and of much lower specific gravity, we must now keep a closer watch than before upon the time of centrifugalisation.

The processes of pipetting off and washing in 1% salt solution are again repeated with the same precautions as before.

The corpuscles have now been practically freed from plasma and we draw off with a pipette the whole of the supernatant fluid.

We complete our operation by carefully mixing up our corpuscles, so as to obtain a uniform mixture of red and white. We do this by rapidly rolling the corpuscle tube between our hands. The centrifugal force which is thus applied carries the corpuscles up the walls of the tube and gives both an up-and-down as well as a circumferential movement.

The corpuscular suspension ought to fulfil the following tests :—

(*a*) When centrifugalised in a capillary tube of uniform diameter the proportion of supernatant fluid to corpuscles ought to be approximately as 40% to 60%. (*b*) Examined under the microscope the leucocytes ought to be seen as isolated elements uniformly distributed among the red corpuscles. (*c*) Mixed in a simple capillary pipette with an equal volume of a foreign normal serum the suspension should not

either immediately or on when kept at 37° C. show to the naked eye any sign of hæmagglutination.

12. Preliminary Work.

The preliminary work will consist in (*a*) the collection of the sera, and where required the making of the pool of normal sera; (*b*) the preparation of the washed leucocytes; (*c*) the preparation of the bacterial suspension; (*d*) the making of the "trial trip"; and (*e*) the getting together of the apparatus and materials, and the arrangement of these upon the working bench. The two last of these have still to be treated of.

13. The making of the "trial trip."

The trial trip consists in putting up by the technique, presently to be described, a phagocytic mixture, incubating this, making films, staining these and examining them microscopically.

It serves to tell us whether we have in our bacterial suspension a suitable number of microbes, *i.e.*, that number of microbes which will give us, in the period which we propose to allot to the incubation of the phagocytic mixture, the desired phagocytic count, or approximately that count.

It further serves to tell us whether everything else in the phagocytic mixture is as it should be. We learn, for instance, whether the leucocytes are in good condition, whether the red blood corpuscles have agglutinated, and whether, in the case where we are dealing with microbes which disintegrate rapidly within the phagocyte, the incubation period ought to be cut down.

It cannot be too strongly emphasised that it is foolhardy to sit down to the laborious task of doing opsonic measurements upon a long series of bloods, without having first made a "trial trip," to see that everything is going right, and that we are obtaining film-preparations such as are shown in Plate V., Figs. 1, 2 and 3.

14. Procedures which may be resorted to when no phagocytosis is found in the opsonic films.

When on examining the films made in the "trial trip" phagocytosis is found absent or quite insufficient, and no better results are obtained by increasing the number of microbes in the suspension, it will be well, before abandoning the estimation, to wash the bacteria thoroughly upon the centrifuge, so as to get rid of all bacterial detritus and matter in solution; and, if that fails, to digest the suspension with many times its volume of the control serum, following, of course, exactly the same procedure in the case of the patient's blood.

15. Collection and preparation of our apparatus and materials; and arrangement of the working bench.

We begin operations by looking to see that the thermometer in the opsoniser is registering blood-heat. If it is not we make the necessary adjustment.

We then go through our capillary pipettes to satisfy ourselves that the capillary stems are sufficiently fine and not too fine, and that the barrels fit the teat. Taking up the pipettes by their capillary stems four or five at a time, we now arrange them palisade fashion between the finger and thumb of the left hand, and then drawing a sharp glass-cutting knife firmly across them, nick them at the

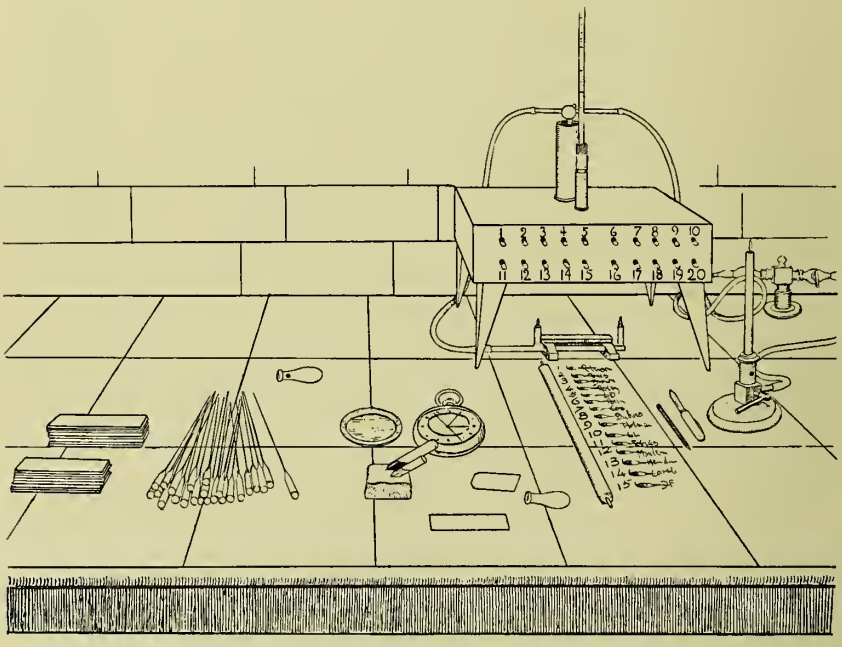


Fig. 62.

level of the forefinger. When this has been done a sharp tap administered with the knife blade upon the ends of the tubes will break them off with a clean square fracture at the level of the nick. If any pipette presents a jagged or irregular extremity it must be put aside and recut. Stemming the barrels of the pipettes against the bench, we now push forward the capillary stems until they project about 2 cms. from beyond the fingers; then, exchanging the glass knife for a sharply-pointed glass-writing pencil, we draw this across the capillary stems close up to the fingers, so as to inscribe upon each a legible division mark. We now lay these aside and take up another set, until we have prepared a pipette for every blood which has to be

tested, and a few to spare; and finally we lay them all down together in a bundle on our left hand (*vide* Fig. 62) with the barrels facing us.

We now deal with the slides. Providing ourselves with two for each blood to be examined, and a few extra ones, we spin them on the bench, as explained in *Chapter VI., Sub-section* 4, to determine which is the convex side; and then roughen this with fine emery paper, as there described (*supra* loc. cit.). This done, we arrange our slides, keeping the roughened side uppermost, in one or more piles upon the bench.

We take also an unroughened slide and lay it down immediately in front of us. This comes into use in connexion with Operation No. 1 when we blow out the contents of our pipettes to mix them.

We next get together the *materials* we require for our test.

We first go over our blood capsules to see that clotting has taken place, and that the serum has separated out satisfactorily. This done we arrange the capsules in a column on the bench as shown in Fig. 62; intercalating the control bloods at regular intervals between the patients' bloods, and writing down the name which corresponds to each capsule on the bench in the exact position which that capsule is to occupy. Then taking up each capsule in turn, we nick it with a glass-cutting knife at the base of the straight limb; break this off, and lay the capsule down again in its place.

We next take a piece of glass tubing and some plasticine, and roll this out until it forms a fairly thick jacket round the tube. The purpose which is served by this rigid roll of plasticine will presently appear. The roll is to be placed in position on the left of the column of blood capsules, and is to be fixed to the bench by light pressure.

We now take some more plasticine, mould it into a cubical block to serve as a pedestal, and then insert into this the tube containing our washed corpuscles, and the tube containing our bacterial suspension, taking care to incline these tubes at an angle corresponding to the angle at which the capillary pipette will be most conveniently held in dipping into the tubes.

Finally, we collect around us everything that we want or may want in the course of our work: to wit, a watch, or stop-watch; a glass writing pencil; a glass-cutting knife; an unperforated and a perforated teat, a well-tested spreader; a watch-glass of water for cleaning the spreader; a Bunsen burner with a peep-flame; and (where we are working with dangerous microbes) a vessel containing an antiseptic.

16. The carrying out of the actual opsonic measurement.

For the purpose of description, the procedure may conveniently be divided into four separate operations:—

1. The making of the "phagocytic mixtures" in capillary pipettes—*i.e.*, the measuring off and mixing of equal volumes of washed corpuscles, bacterial suspension and serum; and the placing of the capillary pipettes in the opsonic incubator (opsoniser).

2. The removal of the capillary pipettes from the opsonic incubator and the making of film-preparations.

3. The fixing and staining of these.

4. The enumeration of the microbic ingest of a series of phagocytes with a view to arriving at the average microbic ingest; and the working out of the opsonic index.

17. Operation No. 1.—The making of the phagocytic mixtures and the incubation of these.

We begin by taking a pipette from the bundle which lies ready on the left side of the bench. This is best done with the left hand, the teat being fitted to the pipette with the right. Then, operating as described in *Chapter IV., Sub-section* 1, we aspirate into the pipette—following the order* in which they are here enumerated—one volume of washed corpuscles; one volume of bacterial suspension; and one volume of serum.

During this operation the tubes containing the corpuscles and bacterial suspension—it will be remembered that they have been sloped to an angle which allows of convenient access—remain on the bench, while the blood capsule is taken up and held in position by the left hand until the required quantum of serum has been obtained. This done the capsule is sealed up by thrusting it into the nearer side of the roll of plasticine, the residue of the serum being thus reserved against future eventualities.†

The three volumes of fluid which have been aspirated into the pipette have now to be thoroughly mixed. This is effected by blowing them out on to the clean unground glass slide, re-aspirating and blowing out the mixture at least six times in succession.

This done the fluid is aspirated into the pipette, taking care to avoid breaking the column by bubbles, and to draw it up far enough to leave the terminal centimetre or two of the stem free.

* If this order is departed from we must carefully wipe off the end of the pipette before bringing it over from fluid to fluid.

† In case of our having to go back upon a serum, this method makes it certain that the proper sequence of capsules has been maintained, and in any case we are insured against the possibility of taking up a blood we have just laid down in mistake for the next in series.

The end is now sealed in the by-pass of the Bunsen burner, and the pipette is inserted into the first tube-compartment of the opsoniser.

We now look at the watch and note down the time upon the bench, over against the name which designates the blood.

In taking the time with the stop-watch we set the hands and put them in motion when we put our first blood into the opsoniser, and read off the time again as each successive pipette is inserted, noting these down on the bench. We then, as soon as the appointed incubation period has expired, bring back the hands to their original point of departure, and take out the successive pipettes from the opsoniser when the minute and second hands have come round again to the positions which they occupied when the pipettes were inserted.

When we employ an ordinary watch we proceed on somewhat similar lines. Dividing the hour into quadrants, we note down in inserting a pipette into the incubator the time which has elapsed since the minute hand entered the quadrant, and we take out the pipettes when the minute and second hands take up the corresponding positions in the next following quadrant.

These operations can with practice be carried through comfortably in somewhat under the minute. One may, therefore, when a series of bloods is to be tested, calculate on getting them into the incubator at the rate of one a minute.

Fifteen minutes is the usual time limit to allow for incubation in the opsoniser. But this period may be prolonged when the bacterial suspension is too thin and gives too small a phagocytic count; and it may be shortened within limits when the trial trips show that a quarter of an hour in the opsoniser gives too large a count.

Considerations other than the density of the suspension may here also be taken into account. When we have to deal with a batch of over 15 and under 20 bloods, it is well, with a view to getting the whole batch done at once, to lengthen out the incubation period correspondingly. In the case where we have less than 15 bloods, and it is matter of importance to save time, we may in like manner cut down the incubation time. But in as much as irregular results would be obtained if the incubation time were unduly shortened, it will be well—except in the case where we are dealing with microbes, such as the gonococcus and certain coliform bacteria, which undergo rapidly digestion in the interior of the leucocyte—not to cut down the incubation period below 10 minutes.

18. Operation No. 2.—Removal of the phagocytic mixture from the opsonic incubator, and making of the film-preparations.

Any unused pipettes which remain over are now rapidly brushed on one side and the pile of prepared slides is brought forward in their place. The ordinary teat is discarded and a perforated one placed ready to hand. A pair of ground slides is now taken from the pile; their upper surfaces are wiped to remove any adhering traces of emery

dust; and we place them side by side in front of us with their edges parallel to the edge of the bench. (Fig. 63.)

As soon as the period appointed for the incubation has run out pipette number one is removed from the opsoniser, and the perforated teat is fitted to it. We now break off the tip of the capillary stem with the finger nail, or if there is any objection to this, snap it off after nicking with a glass-cutting knife; and we then, covering with thumb or finger the hole in the teat, compress it so as to blow out the

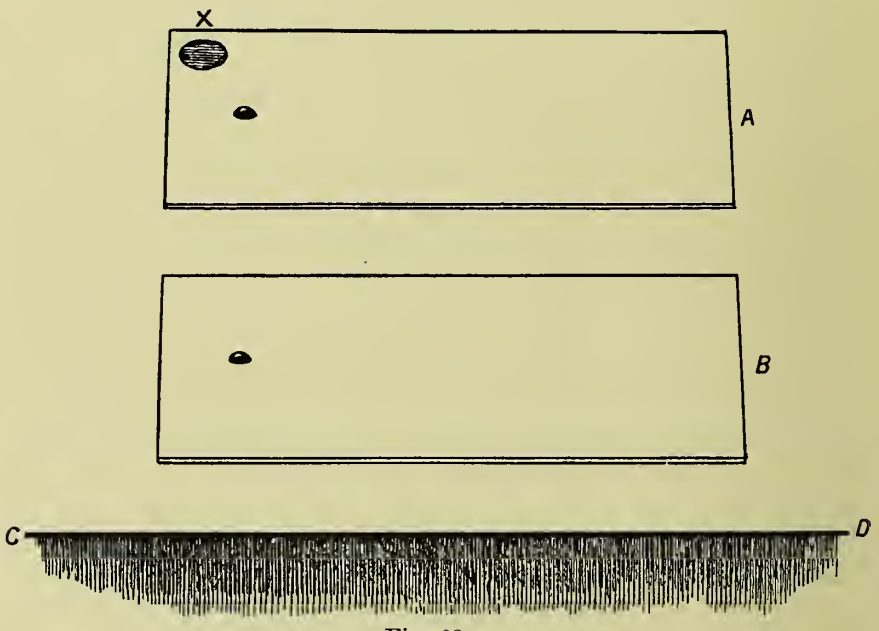


Fig. 63.

contents of the pipette on to the extreme left-hand corner (the point is marked in the figure with an X) of the uppermost of the pair of slides which have been placed in position to receive the films. (Fig. 63.) We next proceed to mix carefully, drawing up and blowing out the phagocytic mixture at least six times. Finally we carry over what we require of the mixture to a point on the median line of the slide, about 1 cm. from its left-hand end, and deposit it there in the form of a convex drop of the dimensions indicated in the diagram by the small circle. We deposit also, a similar drop of the mixture on the companion slide. The spreader is now taken and we make our films by the technique described in *Chapter VI., Sub-section* 5, b,

which brings the phagocytes to the right-hand margin of the preparation. The edge of the spreader should be wiped on a dry cloth after spreading each pair of slides; and from time to time it may be dipped into the watch glass of water, being afterwards carefully dried.

As we make the films we number each pair with the serial number of the blood, inscribing the numbers with a glass-writing pencil on the right hand end of the slide.

We then, with a view to going to work in each case on the better of the two films, and also with a view to saving time when we are assem-

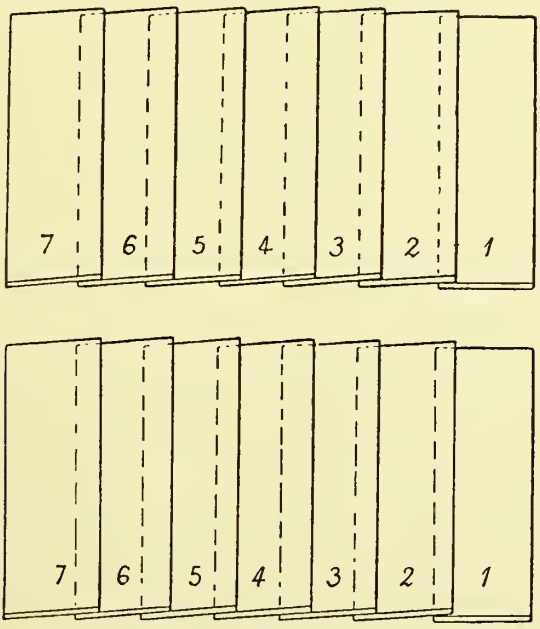


Fig. 64.

bling the whole series of slides for staining, critically scrutinise each pair of films, and then arrange them end to end instead of side by side, the better of the two films occupying the proximal position as we hold them. Preserving this order we lay them down at half arm's length away on our right with their longitudinal axes disposed at right angles to the edge of the bench. Each pair of slides after the first is to be disposed so as to overlap the edges of the previous pair in the manner indicated in Fig. 64.

19. Operation No. 3. — Fixing and Staining of the film-preparations.

The row of slides which has been placed nearest to the edge of the bench—it will be remembered that this row consists of selected slides

—is now collected by placing one hand at each end of the row and bringing them together round the slides. The manœuvre is exactly the same as that by which we collect a pack of cards which is spread out over a table.

Fixation of the films.—We now fix the films with the vapour of formalin or with a saturated solution of perchloride of mercury in water. Both these give excellent results, but there is a balance of advantage in favour of the formalin fixation in the case of films containing tubercle bacilli and microbes which undergo rapid disintegration within the cells, while in the case of films containing non-acid-fast micro-organisms fixation by sublimate has the advantage.

Where we elect to employ formalin fixation we pour a little formalin into a watch glass or salt cellar, and then invert the films over the vessel, holding each film in succession there for the space of a few seconds, taking care to see that there are no drops of formalin on the edge of the vessel which might come in contact with the preparation.

Where we employ corrosive sublimate we spread out the slides upon the staining rack (see Fig. 9, p. 7), pour on the saturated solution, allow it to act on the films for from $\frac{1}{2}$ to 1 minute, and then wash off thoroughly in a stream of water.

Both in connexion with fixation, and in connexion with all the operations of staining, it is essential to securing an exact comparability between the films that they shall be treated in every respect alike, the same reagents being applied to each, and the time of exposure to those reagents being in each case exactly the same.

Staining of the films.—For the purposes of the staining operations "phagocytic films" may be classified into (*a*) films containing microbes which can be stained by ordinary processes, (*b*) films containing tubercle bacilli, and (*c*) films containing microbes which undergo rapid disintegration in the interior of the leucocytes.

We may deal with these seriatim, first, however, disposing of certain details of technique which apply indifferently to all the processes.

Washing is most conveniently carried out on the staining rack, the water being supplied through a hose fitted to the tap (see Fig. 9, p. 7).

When the wash water is to be drained off we bring the slides together edge to edge so as to allow the water to flow across from one to the other, and we then tilt up the edge of the staining rack.

When the staining is to be undertaken the slides are to be spaced out again so as to allow of their being separately flooded.

Where the stain has to be heated, this may be done by applying a flame to the end of the slide as they lie upon the rack; but,

where we are dealing with carbol fuchsin, it is preferable to heat the stain in a large test-tube, afterwards pouring it on to the slides.

(*a*) *Staining of films which contain microbes that can be stained by ordinary processes.*—Any of the ordinary basic stains may be employed, but carbol-thionin (thionin ·25 gramme, 1% carbolic acid 100 grammes) gives on the whole the most satisfactory results. If the carbol-thionin is applied undiluted half a minute will suffice for the staining. If it is applied diluted two or three minutes may be allowed.

(*b*) *Staining the films which contain tubercle bacilli.*—The ordinary carbol-fuchsin solution (fuchsin 1 gramme, 5% carbolic acid 100 grammes) is employed. This is brought nearly to the boil in a large test-tube, and is then poured over the separated slides—sufficient stain being used to give an ample covering of fluid—and is allowed to remain on for ten minutes. It is then washed off with a stream of water, this being applied to the slides in the same order as the stain was put on, so as to make the period of exposure to the stain as nearly as possible alike. The superfluous wash water, which if left on might dilute the de-colourising acid in an irregular way, is then drained away.

A 2·5% solution of sulphuric acid is now poured over the slides, following on rapidly from one slide to another. It is allowed to remain on for about 20 seconds and is washed off with the hose in the same order as it was applied, great care being here taken to equalise as far as possible the period of exposure to the reagent.

The next step is to flood the film with 4% acetic acid. This procedure, which was suggested by my fellow-worker, Capt. S. R. Douglas, removes the hæmoglobin from the red corpuscles—making them invisible—and gives a better differentiation with the methylene blue which is used as a counter stain to the tubercle bacillus. The acetic acid is washed off and then a solution of alkaline methylene blue (methylene blue 1 gramme, carbonate of soda 1 gramme, distilled water 200 grammes) is poured on and is allowed to remain on for about 30 seconds.

It is then washed off, the stream of water being turned off from the films as soon as the general blue colour has been removed. The slides are then taken several at a time, held by their edges between the fingers of both hands, and thoroughly shaken in order to get rid of as much water as possible. They are then dried between sheets of clean white blotting paper, and are finally placed on the top of an incubator or in any other warm place to get rid of the last traces of moisture. All this is done in order to prevent the blue colour being washed out—as it very easily may be from the body of the leucocyte. If even a

trace of water is left on the film a smudge is developed in which the leucocytes are found partially decolourised.

(*c*) *Staining of films which contain micro-organisms that do not hold on firmly to ordinary stains, or which rapidly disintegrate and lose their staining properties in the interior of the leucocytes.*—These films are treated like tubercle films, first with carbol fuchsin and sulphuric acid, and then with methylene blue.*

20. Operation No. 4.—Enumeration of the opsonic film.

In connexion with the counting of the opsonic film we have to consider *first* the *number of leucocytes* which require to be counted; *secondly*, the *technique of counting* and noting down the figures; and, *thirdly*, the *code of morality* which ought to come into application in making the count.

21. Number of leucocytes to be counted.

The theoretical principles which determine the number of leucocytes to be counted in each case are critically considered in an Appendix at the end of this Chapter. What will be feasible to do will be in each case to count the bacterial ingest of several sequences of leucocytes chosen at random; and then, if the counts for these sample groups show a fair agreement, to take an average of the whole.

In the ordinary case it will suffice to take five sets of twenty leucocytes. The counts of such samples will often be found to agree within 10%, but if the counts for the individual groups should differ from each other by 20%—and such wide disagreement ought to make us a little suspicious of our technique—it will be well to count another 50 or 100 or even more leucocytes.

22. Technique of counting and noting down the figures.

Passing now to the technique of counting and noting down the figures, we place the slide upon the stage of the microscope, and clamp that end of the slide which is most remote from the leucocytic edge, leaving the other end free to be moved about under the objective. We then rapidly scan the preparation under the low power of the microscope, to find the thin line of leucocytes which marks the end of the film (as in Plate V., Fig. 1), and select the part where the leucocytes are most numerous, and best stained, and are displayed to best advantage.

The oil-immersion lens is now brought into use, and the end of the slide is moved slowly across the stage of the microscope so as

*This procedure suggested itself to my fellow-worker, Dr. Noon, on reflecting upon the deep staining of the contaminating bacteria in preparations stained for tubercle.

PLATE V.

Fig. 1. The edge of an opsonic film (under a comparatively low power).
showing the leucocytes gathered to the end. Stained with methyl violet and eosin

Fig. 2. A portion of the edge of an opsonic film (under a high power).
showing phagocytosis of staphylococci. Stained with methyl violet and eosin

Fig. 3. A portion of the edge of an opsonic film under a high power
showing phagocytosis of tubercle bacilli. Stained with methyl violet and eosin
counterstained with

Fig. 4. A zoöglœic mass from a tubercle culture (under a high power).

Fig. 5. Enumeration of bacteria in a vaccine. A portion of the prepara-
tion under a very high magnifying power.

PLATE V.

Fig. 1. The edge of an opsonic film (under a comparatively low power), showing the leucocytes gathered to the end. *Stained with carbol thionin.*

Fig. 2. A portion of the edge of an opsonic film (under a high power), showing phagocytosis of staphylococci. *Stained with carbol thionin.*

Fig. 3. A portion of the edge of an opsonic film under a high power, showing phagocytosis of tubercle bacilli. *Stained with carbol fuchsin and methylene blue.*

Fig. 4. A zoogloeic mass from a tubercle culture (under a high power).

Fig. 5. Enumeration of bacteria in a vaccine. A portion of the preparation under a very high magnifying power.

Fig 1

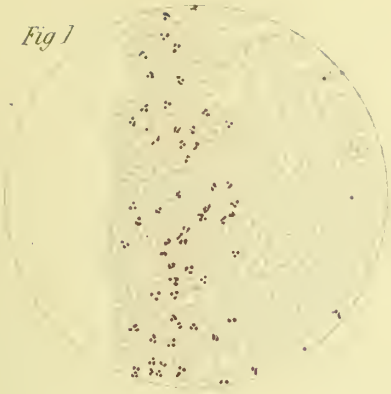


Fig 2.

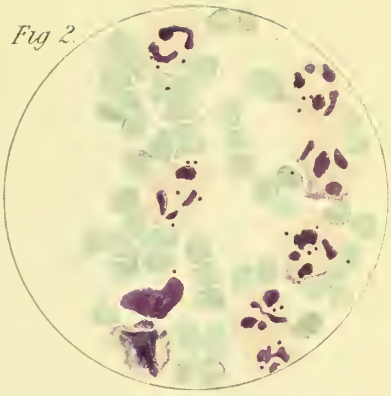


Fig 5.

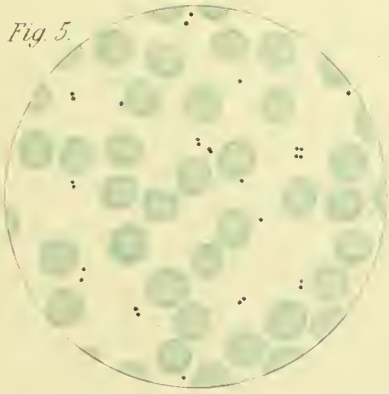


Fig 3

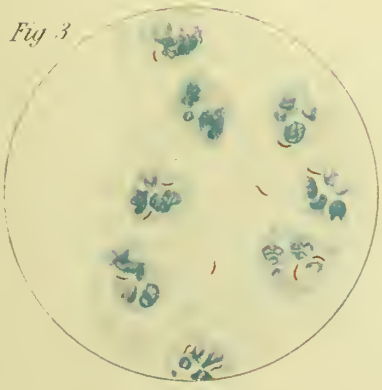
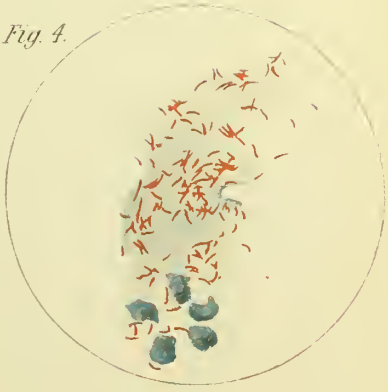


Fig 4.



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to bring a succession of leucocytes along the edge of the film into view. This can be done by hand better than by any mechanical stage arrangement.

The record of the figures may be kept in several different ways.

(*a*) The figure for the bacterial intake of each cell may be written down separately. This method, if it is not to be very laborious, involves dictating the figures to an assistant.

(*b*) We may set down on paper the figures for the bacterial intake of a sequence of cells,—counting as we go along by the *method of vulgar fractions*. In employing this method the number of bacteria ingested furnishes the numerator, and the number leucocytes counted the denominator of the fraction. The numerator and denominator are of course always changing as we proceed; and we stop when the figures become at all unwieldy.

We should, for instance, in dealing with a succession of leucocytes which contained respectively

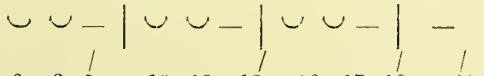
0, 3, 2, 5, 0, 3, 3, 1, 2, 2 bacteria, count

$$\frac{0}{1},\ \frac{3}{2},\ \frac{5}{3},\ \frac{10}{4},\ \frac{10}{5},\ \frac{13}{6},\ \frac{16}{7},\ \frac{17}{8},\ \frac{19}{9},\ \frac{21}{10}$$

A certain amount of practice will be required before this method can be carried out with the necessary degree of certainty, and until that practice has been acquired—and always when there is a risk of one's being addressed or otherwise interrupted in a count—it is advisable to enumerate only short sequences.

(*c*) This method agrees with the last in the respect that we set down figures for sequences and not for individual cells. It differs in the respect that only the numbers of bacteria ingested are here directly enumerated. For the enumeration of the leucocytes we employ a *memoria technica*. We either let the figures for the bacterial ingest fall, as we count them, into a metrical cadence which tells us when we have completed, let us say, 10, or—when twice repeated—20 leucocytes; or we may employ a heel and toe method, punctuating with the toe and heel, first of one foot, and then of the other, as we complete the count respectively of 5, 10, 15, and 20 leucocytes.

In the case of such a sequence of figures as was in question above, we should, using the cadence method, count and scan as follows, using three anapæsts and a terminal accented syllable.



0, 3, 5; 10, 10, 13; 16, 17, 19; 21.

Or, using the toe and heel method, we should punctuate thus:—

0, 3, 5, 10, 10; 13, 16, 17, 19, 21;

23. Method of working out the opsonic index.

As already explained (*supra, Sub-section* 3), the opsonic index is obtained by dividing the phagocytic count of the patient's blood by the phagocytic count of the control normal blood. In the case where several control bloods are employed, the average of the phagocytic counts of all these is reckoned as the normal.

24. Code of morality which ought to come into application in counting opsonic films.

It is a precondition of accuracy in opsonic work that a proper code of morality shall be applied in connexion with the opsonic count. Two different kinds of moral problems here present themselves; on the one hand the problem as to how certain elements—elements which there can be no question of eliminating from consideration—ought to be counted; and, on the other hand, the problem as to whether in making an opsonic count we ought to count every element we see before us, eliminating nothing.

(*a*) *Problem as to how certain elements ought to be counted.*—This problem presents itself where we have in the cell bacterial division-forms or definite diplococci; where we have bacteria lying on the extreme edge of cells; where we have bacteria which we suspect to be lying on the top instead of in the interior of the leucocytes; and where the microbes in a certain percentage of the cells are too many to enumerate.

The proper morality in all these cases is to make up our minds as to how they may best be dealt with, and then to abide consistently by what we have decided. Where a microbe is lying on the border of a cell it will be a matter of indifference whether we count it in or out, provided we are consistent in our policy. The same thing will apply in connexion with diplococci or division-forms. It will be a matter of indifference whether we count each of these as one, or as two. In the case of individual microbes—as distinguished from zoogloeic masses—which may be lying on the top of the cell, the better practice will always be to count these as if they were in the cells; for we have no means of telling definitely whether our suspicions are or are not well grounded. In the case of a beaded or vacuolated bacillus, it will always be better to count the whole element as a unit; and, following the same practice, it will be well in the case of a streptococcus, or any ordinary aggregation form, to count the whole aggregate as a unit. Where we have in some of the cells a larger number of microbes than it is possible to enumerate with certainty—and this is one of those contingencies which the "trial trip" is intended to provide against—we may compromise by fixing a maximum. We

may, for instance, where we are dealing with tubercle bacilli, take 9 as our maximum, for it will rarely be possible to count with certainty more than 9 tubercle bacilli in a leucocyte.

(*b*) *Problem as to whether we ought to count every element, eliminating nothing.*—In connexion with broken fragments of bacilli (such as will be found in badly prepared tubercle suspensions); with any element in a cell with regard to whose identification as a microbe a doubt may arise; and, further, in connection with broken, or badly stained, or agglomerated leucocytes, the question as to whether these ought not to be passed over and left out of the count will inevitably present itself.

This question is the most important question of morality which presents itself in connexion with the phagocytic count. Deliberately to leave things out of the record would at first sight seem to be a direct violation of every moral mode. When, however, we look carefully into the matter, it becomes clear that the policy of leaving things out of the record may under certain given conditions be the inevitable and only proper policy.

Let us, for instance, call up before our minds the case that would present itself if we were set to count the average number of pips in a pack which contained cards which were defaced in such a manner that the markings could not be identified and counted, and others from which large pieces had been torn off. Here it will be clear that the only way to arrive at a true average for the original undamaged pack would be to reject every card which was defaced or defective; provided always that, in entering a card upon the record, or rejecting it, we remained quite uninfluenced by the consideration that the inclusion or rejection of the card might raise or lower our average, or might, in the case where a series of sample batches of cards were being counted, tend to equalise or to introduce an inequality into those counts.

We have here the principle which we must take as our guide in connexion with opsonic counts. It is not only permissible, but imperative—for it is essential to the accuracy of the count—to leave out of the record any element in connexion with which an uncertainty arises, provided always that our decision as to its admission or rejection is uninfluenced by conscious or unconscious bias. If this were not justifiable, the trial trip, and in particular the preliminary survey of the film for the purpose of choosing the best area to count, would both be unjustifiable.

It is not enough to have arrived at the abstract principle which governs the case. We shall not be in a position to draw up rules to guide us in our work, until we have envisaged the problem from all its different sides.

Perhaps the two most important questions which we require to have answered are:—

The question as to whether there will be found in every opsonic film elements which it will be well to leave out of the record; and as to what precautions ought to be taken to avoid elements being eliminated from the record under the influence of conscious and unconscious bias.

The question as to whether there is any limit to the number of elements we may reject; and as to wherein, if we may reject unsatisfactory elements without any limitation, lies the advantage of working with a good instead of a bad film.

(*c*) *Question as to whether there will, in every opsonic film, be elements which ought to be left out of the count.*—In connexion with this question, consideration will show that a great deal will depend upon the technique of the particular operator. With ideally good technique there will not be any torn or agglomerated, or badly stained leucocytes. And with ideal technique, and a culture and microbe which lend themselves to the purpose (*i.e.*, with a culture which can be broken up with ease and a microbe which resists digestion and can be stained so as to stand out clearly in the cell), there will not be any broken fragments of microbes, or agglomerated masses or badly stained bacterial elements. When these conditions are not satisfied, there will always be a certain number of doubtful bacterial elements. And with technique which falls short of the ideal, there will, of course, always be both leucocytic and bacterial elements which had better be left out of consideration.

These things being so, it will always be counsel of perfection when we are counting films derived from a patient in whom we have a personal concern, or films upon which an important scientific fact rests, to get a fellow worker to number the films and keep a key, and then to hand them over to us to count. But it will be well to understand clearly that with real good films, and good suspension of a suitable microbe, such as the staphylococcus or the tubercle bacillus, it would be very difficult under any circumstances to go seriously wrong.

(*d*) *Question as to whether there is any limit to the number of elements we may reject.*—Coming to the question as to whether there is any limit to the number, or rather to the percentage, of elements we may reject on the ground of ambiguity and bad definition, it might plausibly be argued that, once the principle of omitting anything from the record has been admitted, it is logically impossible to draw a line anywhere; and that it would be as legitimate to reject 95 per cent. as 5 per cent. of the elements in an opsonic film.

This argument would be sound if only it were practicable to place every element which we meet with in an opsonic film, either in the class of *absolutely clear and unambiguous elements*, or in the class of *plainly indistinct and ambiguous elements*.

In reality, however—and for this reason it is advisable to count anonymous films whenever our technique leaves anything to be desired—there is an intermediate class of, we may perhaps call them, *doubtfully distinct elements*. And this class will be relatively small when the film contains few "plainly indistinct and ambiguous elements; and it will be relatively large when the film contains many elements which would fall into that class.

We can see that this must be so if we take the more familiar case of the haemocytometrical count. Here, if the rulings are distinct and the corpuscles are well defined, there will be no elements which ought to be omitted from the record, and no elements in connexion with which a half-doubt will arise in the mind. But if we are dealing with a haemocytometer slide whose rulings have been partially obliterated, or if our corpuscles are badly defined against the background, there will, in addition to elements which must obviously be excluded from consideration, be a relatively large number of elements, in connexion with which we are a little doubtful.

Now it is precisely in connexion with the class of elements concerning which we are just a little doubtful that uncertainty and fallacy come into our haemocytometrical counts and our opsonic counts.

And the real outstanding advantage of good technique consists not so much in the fact that it eliminates the class of "plainly indistinct and ambiguous elements," but in the fact that it eliminates the class of "doubtfully distinct elements."

25. Methods by which we may determine whether a conformity or difference, which we have found between two bloods, corresponds to a genuine agreement or difference between these, or is to be attributed to a working error.

(1) *Evaluation of the working error in the case where we have duplicate controls.*—Where in connexion with a tuberculo-opsonic measurement, several different control bloods, or in connexion with any other opsonic measurement, duplicate specimens of the same control blood, have been taken and tested, we have under our hand all that we require for the evaluation of our working error.

If our controls disagree this will cast suspicion on the whole of our results. If they show close agreement we may—provided always that our bacterial suspension has proved itself to be a sensitive indicator of differences in opsonic power (*vide supra, Sub-section* 8, *d*)—conclude

that our working error is of small dimensions, and need not come into account.

(2) *Evaluation of the working error in the case where duplicate controls are not available.*—In the absence of duplicate controls it will still be possible to evaluate our working error by evaluating its components: *our functional error*, and the *probable error of chance.*

(*a*) *Method by which we can evaluate our functional error.*—We can estimate the dimensions of our functional error by introspection and self-examination, followed up by a critical scrutiny of our opsonic films.

α. *Introspection and self-examination.*—Every man who sets up before himself any standard of efficiency will know the feeling of having done his work well, of having kept his attention concentrated upon his task, of having been meticulously careful in every stage of his operations; and he will also know how it feels to have failed to exercise sufficiently strict supervision over himself, to have been guilty of momentary lapses of attention, and to have come short in the matter of keeping intellectually alive when doing his work.

All this will hold true in a quite pre-eminent manner of the laboratory worker in connexion with opsonic measurements. He will quite well know whether he has measured off his unit volumes accurately; whether he has mixed sufficiently carefully; whether he has kept the record of his count with sufficient accuracy; has, in eliminating elements from his count, acted always with judicial rectitude—shutting off from himself wherever this is called for all opportunity of bias; and has consistently applied one and the same rule to all cases of ambiguity, such as arise when a bacterial element might be counted as in or as out of a cell, or as one or as two.

If he feels that he has conformed to this code of intellectual morality, if he has exercised wariness with respect to the fallacies which have been set out in the foregoing; and if his film preparations will sustain critical scrutiny, he may rest assured that his functional error will not be such as seriously to affect his results.

β. *Critical scrutiny of the opsonic film.*—Consideration will show that while one portion of our work—to wit, the measuring off of the unit volumes of our reagents, the mixing of these, the timing of the incubation periods, and the recording of the count—can be reviewed only by introspection and self-examination, there is also another portion of our work which can be enquired into by much more objective methods. A critical scrutiny of the film preparation upon which an opsonic count has been based, will tell us nearly all we require about the bacterial suspension, and all we require with respect to the making of the film preparations, and the staining of these. In fact, it will

tell us so much that if, for the purpose of judging another man's work, his film preparations only were sent up we might, on the presumption that he who has faithfully carried out the technique in all the points that come under review in the film will have been faithful also in all other points, safely conclude, if we found his films in all respects satisfactory, that his working error was very small. If, on the other hand, the films which were furnished to us resembled that reproduced in Plate III., Figures *f* and *g*, we should be entitled, without any further enquiry, to conclude that his working error was very large; for in such films the corpuscles are always ruinously torn in some places and not flattened out in others; and by reason of its varying thickness such a film does not admit of even and regular staining.

(*b*) *Method by which we can evaluate the probable error of chance.*—The error of chance—which may be introduced into our results by the fact that we may, in counting one blood, alight upon a series of leucocytes which would send up the average for the bacterial ingest of that blood unduly, and in counting another blood upon a series of leucocytes which would send the average for that blood down unduly—cannot, as we shall see (*infra*, Appendix to this Chapter), be computed once for all. Therefore, if we are not to involve ourselves in an elaborate process of computation in connexion with each blood, all that will be possible to do will be to compromise by comparing, as suggested above (Sub-section 21) the counts for a series of groups of leucocytes, and when these go apart, by counting further groups and then taking an average for the whole.

But the really essential point which we have to keep in view in connexion with the error of chance, is that it is of altogether subordinate importance to the functional error, and that the dimensions of the error of chance will, for the same number of cells counted, always stand in relation with the magnitude of the functional error,—the probable error of chance being large, when the functional error is large; and small, when the functional error is small.

In correspondence with this we find, in the case where duplicate controls are available, that when our functional error has been kept well within bounds, the probable error of chance on a hundred leucocytes counted is so small as not to come seriously into account. There can be no doubt that this obtains also for the case we are now discussing, the case where duplicate controls are not available. On the other hand, where the functional error is large, it will not be worth while investigating the dimensions of the probable error of chance, for the results arrived at will already have been shown to be unworthy of confidence.

26. Question as to what inferences we shall be warranted in drawing from our opsonic measurements in the case where we have satisfied ourselves that our working error falls out of account.

We have here to consider three different cases.

(*a*) *Case in which only a single opsonic measurement is available and that measurement shows either an exact conformity between the patient's blood and the normal control, or a definite divergence from this.*

(*α*) A single conformity with the normal control will in no case have any diagnostic significance; for even in the extreme case where the patient's blood is fluctuating between high and low opsonic indices, the blood will necessarily from time to time be passing across the normal line.

(*β*) A definite divergence from a normal control or a pool of normal bloods will in the case where we are dealing with a microbe upon which different normal bloods exert very unequal opsonic effects, have no diagnostic significance, or at least no assured diagnostic significance.

In the case where we are dealing with the tubercle bacillus, or any other microbe upon which the blood of every healthy normal adult exerts approximately the same opsonic effect both a definitely subnormal and a definitely supernormal index will furnish presumptive proof of infection by the microbe in question,* or, it may be of infection by a closely allied microbe.†

Such presumption will, however, be infinitely stronger in the case where we have a supernormal index than in the case where we have a subnormal index.

For a *supernormal tuberculo-opsonic index*—and what applies to tubercle applies also to all other microbes which we here have in view—does not in practice come under observation, except where an elaboration of protective substances has been elicited by a tubercular auto-inoculation, or an inoculation with tubercle vaccine.

A subnormal tuberculo-opsonic index—while it will, as a rule, indicate that protective substances have been used up under the influence of a tubercular infection—not unfrequently comes under observation in connexion with syphilis—in particular inherited syphilis. And it would seem that it may also be obtained in connexion with quite

* A statistical analysis of a total of 610 consecutive opsonic measurements of control, normal bloods, chosen at random from the laboratory records of the Department for Therapeutic Inoculation of St. Mary's Hospital, has shown that an index of below 0·8 or above 1·2 found in working with these would have furnished a probability of 9—1 in favour of abnormality with respect to tubercle.—*Harrey and McKendrick, Biometrika, Vol. VII.*, 1909.

† We may, for instance, diagnose leprosy by these means.

temporary constitutional disturbances. Further, an all-round reduction of the opsonic power has not unfrequently come under observation in the last stages of generalised streptococci infections. And finally, as my fellow-worker, the late Dr. J. H. Wells,* pointed out, the opsonic power is often quite undeveloped in infants of a few months old.

(*b*) *Case where a series of opsonic measurements is available, and these show absolutely either no fluctuation or, as the case may be, a very definite fluctuation.*—If no fluctuation is obtained, we may infer with probability that the particular infection whose presence we had suspected is absent; and the presumption in favour of that particular infection being absent will be very strong in the case where there is clinical evidence that auto-inoculations must be taking place; and, in the case where we have vigorously applied procedures which might reasonably be expected to carry a sufficient lymph stream through the focus of infection, to elicit an auto-inoculation.

If the opsonic indices go widely apart, the diagnosis of infection by the microbe which we are employing in our tests is assured. And we shall here, in the case where we have not resorted to any means for producing an artificial auto-inoculation, have evidence that the patient is undergoing "spontaneous" auto-inoculations; and in the case where we have been employing a procedure for artificially eliciting auto-inoculations, we shall be entitled to conclude that the particular region of the body which has been searched by our procedure is the seat of infection.

(*c*) *Case where our opsonic measurements have not given a decisive result.*—It will be well to recognise that this case will be constantly presenting itself, in particular in those cases where the investigation has been limited to a single measurement of the opsonic index. In such a case every man who has any gift of common sense will, instead of asking from the opsonic method more than it or any other method is capable of giving, repeat the test upon further specimens of his patient's blood, or if he has up to this confined his search to a search for spontaneous variations in the patient's blood, he will now have recourse to procedures for evoking auto-inoculations.

And, of course, concurrently with his opsonic measurements—and all the more in cases where these do not immediately resolve the problem—the practical doctor will take into account every other feature of his case which may help to throw light upon its diagnosis.

* "Practitioner," May, 1908.

APPENDIX TO CHAPTER IX:

A Consideration of the Contention of the Mathematical Statistician that he has Authority to pronounce Judgment upon the Number of Leucocytes which require to be counted in the Opsonic Film.

The measurement of the opsonic index differs, as the reader will have appreciated, from all the other quantitative methods of blood examination which are described in this book in the respect that there enters into it a factor of chance.*

Instead of counting all the leucocytes in a phagocytic mixture and basing our phagocytic count upon a complete census, we take at haphazard a certain number of leucocytes, determine the average ingest of these and take it to represent the average ingest of the whole "population" of leucocytes.

While the legitimacy of this procedure cannot be called in question, not a few doubts have been expressed as to whether the conditions for a legitimate use of this method—this method, which is employed unreproved in connexion with the hæmocytometer where we count only 10 squares and multiply our error by anything up to 10,000—can be found in connexion with phagocytic films. That is to say, doubts have been expressed as to whether the working error of the opsonic method is not such as to demand the enumeration of a quite impracticably large number of cells.

The decision of this question rests—so the mathematical statistician informs us—with himself; it is for him to say how many leucocytes we must count, and it is for him to tell us what will be the error attaching to phagocytic counts based on the enumeration of the bacterial ingest of 25, 50, 100, and more leucocytes.

It will clearly be advisable to examine these claims, and we may best work up to the complicated problem which presents itself in the opsonic film in a series of stages.

The simplest case which can present itself in connexion with the computation of an average for a whole population, from the examination of a sample, would perhaps be the case already adverted to in *Sub-section* 24, *b*, which would be before us if we were presented with a well-shuffled pack of special cards, and were asked to find out the average number of pips per card, the cards being too numerous to allow of our going through them all.

* This factor of chance enters, be it noted, also into the method which is described in Chapter XI., Sub-section 6 for the enumeration of the number of microbes in a vaccine.

We may at once dismiss the case of the pack furnishing, when sampled, only cards having the same number of pips, for here there would be no error of chance, and the average arrived at would for all purposes of practical life be the same whether we counted 25 cards taken at random or 1,000.

The case we are really concerned to examine is the case where there are two packs: one in which—as shown by sampling—the cards are furnished, let us say, either with 0, 1, 2, or 3 pips; and the other in which the cards are furnished with either 0, 1, 2, 3, 4, or 5 pips, the frequency curve being approximately similar in both cases, and both packs properly shuffled.

It will now be clear that if we enumerated only a small number of cards out of each pack, we might in the one case alight on a series of cards which would send the average for that pack up unduly, and in the other case upon a series of cards which would send the average for that pack down unduly. It is, of course, an error of this sort which we have in view when we speak of a *probable error of chance*.

Further, it will be clear that it would be possible to compute the magnitude of the probable error which would attach to counts of any particular number of cards selected at random; and to fix for each pack the number of cards which it would be necessary to count in order to bring down that error to quite insignificant dimensions.

A result thus arrived at would clearly be binding in connexion with every similar pack of cards, and if in connexion with any such pack less than the full number of cards that were required for the practical elimination of the error of chance were enumerated, and an average computed upon that smaller number of cards, the results would be subject to a fixed correction for probable error due to the operation of chance.

For us, however, it will not do to stop at this point. It behoves us to note that if the calculation were made for the first pack of cards we should, if we counted in the second pack only the number of cards required for the first pack, be counting too few; and, contrariwise, if the calculation was made for the second pack, and we counted in the first pack the full number of cards required in the second, we should be counting unnecessarily many.

This is the case which presents itself in connexion with opsonic measurements. For we have not to deal with bloods which have all a normal index, and with phagocytic mixtures which contain always the same number of similar microbes. We have to deal with bloods which have both high and low opsonic indices, and with phagocytic mixtures which contain quite different numbers of not always similar microbes.

It cannot, therefore, be legitimate to prescribe for the purpose of eliminating the error of chance the counting of one particular number of leucocytes in all cases. Nor can the magnitude of the probable error of chance which would be imported into the counts by divergence from a prescribed standard be the same in all cases.

There is, in connexion with these prescriptions and requirements of the mathematical statistician, a further point which it will be well to make clear at the outset. This point relates to the terms "significant" and "non-significant." In speaking of a difference which has emerged between two groups of figures the mathematical statistician uses the term *significant* when he means that his calculations have shown that there is a large balance of probability in favour of that difference being due to causes other than the operation of chance; and he uses the term *non-significant* when he means that the operation of chance might quite well account for the difference.

Let it, however, be observed that the term "significant"—and what applies to it holds of course also of its correlative "insignificant"—may carry also a second meaning. When used in connexion with a phagocytic count of a patient's blood it may quite well import not only that the difference between it and the control does not resolve itself into a working error, but also that it is one which warrants us in diagnosing the presence of infection.

But even apart from this ambiguity, the terms "significant" and "insignificant" carry, to the ordinary reader, fallacious implications. They suggest that there is to be found somewhere a sharp dividing line between what is probable and what is improbable, and between what is worth taking into consideration and what is not worth taking into consideration; and they suggest to the ordinary reader that the mathematical statistician who uses the terms has scientific resources at his disposal which justify him in affirming with respect to differences which fall within certain limits that they *are* due to the operation of chance, and in dictating that such differences—even though they may be constantly recurring in significant connexions—should be simply wiped off the record and disregarded.

It cannot be too clearly understood that the mathematical statistician has no such secret wells of wisdom to draw from, and that his science does not justify his going one step beyond the purely numerical statement that—as computed by him from the data he has selected as suitable for his purposes—the probabilities in favour of a particular difference being or not being due to the operation of chance are such and such.

There need, therefore, be no hesitation in saying that when the mathematical statistician makes free with the terms *significant* and

non-significant, he is simply taking upon himself a function to which he can lay no claim in his capacity of a mathematician.

It is his proper function to compute the probabilities, it belongs to the practical man of affairs—in connexion with the opsonic index, to the practical doctor—to decide whether a particular degree of probability may, for the purposes of practical life, be allowed to rank as a certainty, and whether another degree of probability—according as it may be met with once or repeatedly, and in non-significant or significant connexions—is to be left out of account, or be carefully considered.

In reality, the arm-chair statistician has no special qualifications for judging what degree of probability may, or may not, be taken as a safe guide in practice, and what degree of probability may, or may not, be capable of giving valuable hints to a practical worker. Thus, for instance, in the mathematical papers which have concerned themselves with the opsonic index, probabilities of less than 9 to 1 practically never receive attention, and Mr. Major Greenwood goes so far as to hint that a probability of 15 to 1 ought perhaps not to be accepted as significant. From my own practical experience, on the other hand, I should say that when confronted with a grave and undiagnosed case of bacterial infection, one ought to regard a probability of 9 to 1 as an equivalent to a certainty, and a probability of 1 to 3 or even less as something worth going to work upon.

Resuming the thread of our argument after this digression, let us now take for consideration, instead of the simple case which presented itself for consideration in connexion with the packs of cards, the more complicated case of a hæmocytometric count. Here, in computing the number of red corpuscles in the cubic millimetre of blood from the average number of corpuscles found on a series of squares taken at random, we have to take into consideration not only the operation of chance which will give us on one square more and upon another fewer corpuscles, but also the effects of imperfect technique. For clearly, if in measuring off the blood too much or too little has been taken, or if the operation of diluting and mixing has been imperfectly carried out, or if the cover glass which covers in the cell sags in the middle, or if no proper system has been adopted for the enumeration of the corpuscles which fall across the rulings, or if in connexion with the enumeration of the white corpuscles the staining is imperfect, or the illumination of the microscope improperly adjusted for bringing these into view, or if in any other way the proper technique has been departed from either in the carrying out of the count (or in the construction of the apparatus), this will give us a different number of corpuscles in the square, and with that a different average. We are here, as will be seen, face to

face with conditions which are very different from those in the case where we were dealing with packs of cards. There our working error was merely the error introduced by the operation of chance, here our working error is made up of two components: one the error of chance, and the other the functional error of the operator.

Now while, as we have seen, the error which is due to the operation of chance will for bloods which contain similar numbers of corpuscles be invariable, the error which comes from errors of technique will be variable and personal to the particular operator. With a bad technician there will be large divergencies between the numbers of corpuscles on the square, and it would, to get a correct average, be necessary to count large numbers of squares, or even to go over the whole procedure several times. With a good technician it would not be necessary to count anything like as many squares, and with an ideal technician the functional error would disappear; the working error would be simply the error which is introduced by the operation of chance; and the number of squares to be counted merely the number of squares requisite for the elimination of that error.

It will be clear that it would not be conformable to any principle of logic to compute the number of squares required for the elimination of the functional error of the bad technician and to impose upon the good technician the burden of counting these squares. The more logical method of proceeding would be to compute the minimum number of squares required for purging the count from the error of chance, and, having prescribed the counting of this number of squares upon all workers, to impose upon every technician the additional burden of counting as many more as might be required to compound for his personal errors.

We pass now to the opsonic film. It may be well to begin by realising the nature of the task we undertake when we set ourselves to work out the average ingest of a population of phagocytes. We shall best get an idea of what we have to do if we compare what has to be done in the case of the opsonic film with what has to be done in the case of the pack of cards and the hæmocytometrical count. In the case of the cards we have simply to enumerate large features clearly imprinted on quite separate and discrete pieces of cardboard. In the case of the hæmocytometric count we have to distribute the elements which are to be enumerated evenly over a surface which has been divided up for us in squares, and the only complication which arises—for all the really difficult work has been done for us by the skilled instrument maker—is that we have, in connexion with the corpuscles which lie upon the rulings, to adopt some system in counting them in or out, and that where we are dealing with white corpuscles we have to

make them stand out clearly from the background. In the case of the opsonic count, we have, as has been explained at length in the foregoing chapter, first, by breaking up the bacterial masses in the culture, to make for ourselves the separate elements we propose to count; we have then to provide ourselves with the required white corpuscles, keeping these carefully separate; then we have to mix these in measured quantities with the bacteria, and with the serum which induces the phagocytosis. When phagocytosis has occurred we have to collect the leucocytes to the end of the film—spreading each of these spherical bodies out flat so that its bacterial ingest may be brought into view—to stain the bacteria differentially; to stain the substance of the leucocyte so as to tell whether the micro-organisms lie within or without it, and we have the required enumeration then to make.

It will be clear that, even if the leucocytes in our phagocytic mixtures had all exactly the same phagocytic avidity, the greater complexity of this technique and the larger functional error which infallibly goes along with this, would furnish a reason for enumerating disproportionately more leucocytes in a phagocytic film than squares in a hæmocytometer cell.

But the principle which holds of the hæmocytometrical, will hold also in connexion with the opsonic count. There is no logical principle to compel anyone to pay a ransom for the incompetence of others by himself counting more cells or squares.

In the opinion of the mathematical statistician all this will be beside the mark. "Have I not," thus, no doubt, will run his mental comment, "specially gone out of my way to guard against such absurdity being imputed to me by basing my computation of the number of leucocytes which require to be counted upon data in which the functional error of the operator has been brought down to its absolutely irreducible minimum."

The good intention may be very freely conceded, but the statistician has perhaps overlooked the fact that he does not, in his capacity as an expert with figures, possess—and his record proclaims this truth aloud—any greater qualification for deciding what is good or bad technique than for deciding what measure of probability may safely be taken as a guide in practical medicine.

And, perhaps, it is permissible to put forward, in conclusion, the two following general considerations:—

(*a*) That it must surely be futile to issue instructions to practical workers—who have, by frequent sampling of their work, found that the counting of 100 leucocytes gives an average which is very seldom sensibly altered by counting more—that they must, henceforth, either count an impracticable number of cells or give up the opsonic

method—such instructions being based upon an analysis of the counts made by a worker whose credentials are unknown to, or not accepted by, those who are asked to discredit their work in favour of his.

(*b*) The handing over of the adjudication of medical results to the lay mathematician has in the past produced deplorable effects. Coming in as he does into medicine not only without any appreciation of the magnitude of the functional errors which attach to ordinary medical diagnosis, and setting up as he has done a Utopian standard of accomplishment and certitude for biological work, he in reality makes common cause with those clinicians who, while they take no account of the enormous working error of clinical methods, set up for laboratory methods a fantastic standard of infallibility.

CHAPTER X.

ON THE BORDET-GENGOU DEFLECTION OF COMPLEMENT REACTION, AND THE WASSERMANN TEST.

Introductory—Preliminary elucidation—Principles which come into application in deflection of complement tests—Requisites—The differentiating antigen—The indicator antigen—Sera which are required when we decide not to use sheeps' red corpuscles as our indicator antigen—Working instructions—Method of reading off and interpreting the results.

1. Introductory.

The Bordet-Gengou *deflection of complement reaction* and its various applications—among which would be its application to the diagnosis of syphilis (*Wassermann's reaction*)—might perhaps properly have been excluded from consideration in this work, on the ground that these methods of blood testing fall entirely outside the sphere of the author's research work.

None the less, they may be briefly considered here, first, because their omission would leave a very obvious gap in a book which concerns itself in so large part with methods of blood examination; and, secondly, because the *technique of the teat and capillary tube* and, I may add, *tubule* finds, as my fellow-worker, Dr. Alexander Fleming, has shown, a very useful application in connexion with these tests.

It is not proposed here to enter into any minutiæ or to give any detailed instructions with regard to the carrying out of the tests.

2. Preliminary elucidation.

It is now firmly established that the blood of an immunised animal contains (*a*) an antitropic element—*immune-body or amboceptor*—which has a specific chemical affinity for the particular antigen against which the animal has been immunised; and (*b*) a non-specific antitropic element—the *complement*—which acting in conjunction with the immune body exerts a disintegrative action upon the

corresponding antigen. The complement is present in normal bloods and—in contrast with what holds true in the case of the immune body—is destroyed at a temperature of 55°—60° C., and gradually disappears from the blood on standing.

It has been established further that blood when deprived of its complement by heating can no longer break up the antigen. It can no longer, where it is a question of hæmolysing red blood corpuscles, hæmolyse these; or, where it is a question of killing and dissolving typhoid or cholera microbes, kill and dissolve these.

It has been further shown by Bordet that when a blood which contains a specific antitropin is digested with the corresponding antigen its complement is withdrawn. This is shown by the fact that the power of breaking down any other antigen is lost, even though the immune body which would be required for this purpose is present in the blood, or is specially added.

Blood which has been inactivated in this way with respect to red corpuscles, which it would otherwise have hæmolysed, is said to have been *inactivated by deflection of complement.*

Complement is withdrawn not alone in the case where antigen combines with immune body; but also when bacterial cultures, or other particulate matter in fine suspension, or certain chemical substances—such as the so-called *lipoid extracts* which are employed in connexion with the Wassermann reaction—are added to the blood.

The deflection of complement which is obtained with large additions of bacterial suspensions and lipoid extracts is non-specific and without diagnostic significance.

Where, however, in connexion with the addition of a bacterial suspension, we find that the patient's blood gives deflection of complement with a smaller addition of antigen than anyone of a series of normal bloods, this is of diagnostic importance; and indicates that we are dealing with a patient who has undergone inoculations, or auto-inoculations, with the particular species of microbe in question. It is this which constitutes the *Bordet-Gengou test.*

In like manner when on adding *lipoid extract* to the blood we find complement withdrawn by a smaller addition than in the case of the normal blood we have obtained the *Wassermann reaction.*

Followed up only a little further, the principle that additions of bacterial antigen or lipoid extract produce a proportionately greater deflection of complement in the infected would seem to lead on inevitably to quantitative methods of testing with standardised antigens for the purpose of gauging the severity of an infection and following up its progress.

But here the foundations upon which we build, and in particular the foundations upon which we build in connexion with the Wassermann reaction, are as yet only insecurely laid. In point of fact, it is not yet clear whether the deflection of complement which we obtain in connexion with the Wassermann reaction is to be regarded as a *reaction of infection—i.e.*, as evidence of a specific intoxication—or as a *reaction of immunisation.*

In favour of interpreting it as a reaction of immunisation would be the fact that—like Widal's reaction in typhoid fever, which is such a reaction—it is not obtained in the early stages of the infection, and that in the case of severe infections it may be obtained only after treatment is becoming effective. It may, as has been suggested, be a reaction of immunisation to poisons liberated in the organism by the disintegrative changes set up by the spirochæte pallida; and this view would be consistent with the fact that the reaction is obtained also, in a somewhat modified form, in lepra and trypanosomiasis.

3. Principles which come into application in deflection of complement tests.

We have seen that when an addition of an antigen is made to a serum which contains an immune body to that antigen, the whole of its complement will be absorbed, with the result that the serum will become inert with respect to any other antigen that may be subsequently added, even though an immune body which is antitropic to this second antigen be present. This second antigen would in practice consist of red corpuscles, for these according as they failed to dissolve or be dissolved would indicate to naked eye inspection that absorption of complement had, or had not, taken place. We shall find it convenient—for by this means we shall be able to avoid ambiguity and difficult periphrasis—to speak of the first antigen as *the differentiating antigen* and of the second antigen as *the indicator antigen.*

4. Requisites.

The requisites for carrying out the deflection of complement test may be classified under the headings of (1) *apparatus*, (2) *materials required for the test.*

(1) The *apparatus* will consist of graduated pipettes for making the required dilutions and volumetric measurements, tubules set up in a plasticine tray, and a slab of plasticine to fit over these as a lid.

(2) The *materials* will consist of the following:—

Where we avail ourselves of the complement and immune body for sheeps' corpuscles which are contained in human serum we

shall require (*a*) the differentiating antigen, (*b*) the indicator antigen, (*c*) the serum which is to be tested, and (*d*) the control sera.

Where we do not avail ourselves of the complement and immune body for sheeps' corpuscles which are native to human serum, we shall require as before the differentiating antigen and the indicator antigen, the serum which is to be tested, and the control sera—but all these sera will now have to be decomplemented by heating to 56° C. And we shall further require (*e*) a decomplemented serum which contains an immune body antitropic to the indicator antigen, and (*f*) a serum which contains complement but no immune body.

5. The differentiating antigen.

The nature of this will, of course, be determined by the nature of the diagnosis we have to undertake. Where we want to ascertain whether we are in presence of a particular bacterial infection we shall of course use as our differentiating antigen a suspension of the bacteria which are under suspicion. The success or failure of the method—so far as it does not depend upon the regulation of the quantity of antigen—would seem to hinge upon some special and, up to the present undefined, condition of the culture. This holds true in an altogether pre-eminent manner of tubercle cultures ; and it may be noted in passing that none of the ordinary tuberculins—*i.e.*, extracts of autolysed, or suspensions of dried or powdered tubercle bacilli—which are on the market, would seem to fulfil the requirements of a differentiating antigen for tubercle diagnosis.

In the case of the Wassermann reaction, the differentiating antigen originally employed by this investigator—on the idea that he had here the equivalent of the bacterial suspensions of Bordet's experiments—was an extract of the liver of a syphilitic fœtus. And, though the idea which originally suggested the use of this extract has had to be abandoned, the extract is still recommended by Wassermann as being superior to all others.

Since, however, it has been demonstrated that as satisfactory results in the diagnosis of syphilis, or results which are nearly as satisfactory, can be obtained by the use of an alcoholic extract of a normal liver or other organ, nearly all laboratory workers now employ such extracts—that most in favour being an alcoholic extract of human heart. Such extracts are commonly spoken of as *lipoid extracts* since they contain cholesterin and other substances which are chemically related to the fats. Inasmuch as they are alcoholic extracts they must be diluted at least five-fold before use.

Method of proving and standardising the differentiating antigen.—No reliance can be placed upon the results of any deflection of com-

plement test unless the differentiating antigen has first been proved and standardised.

The accepted method of putting an antigen to the proof is to take specimens of bloods from a number of infected persons and also from a number of persons who can be guaranteed free from infection, and to test the whole batch with graduated quantities of the antigen. If it then comes out clearly that deflection of complement is obtained in the infected bloods with smaller additions of the antigen than with the normal bloods, the antigen may be accepted as satisfactory. And the larger the difference the more satisfactory the antigen.

While this procedure proceeds on lines which must be pronounced to be sound in the main, a certain doubt must none the less attach to it. For, if deflection of complement represents a reaction of immunisation as distinguished from a reaction of infection, it follows—and this was indicated above—that some of the infected must fail to give the reaction.

When the quantity of antigen which will give deflection of complement with bloods of infected persons, and which will never give it with bloods of the uninfected has been elicited, this will be the dose to employ in carrying out the test. But in the very nature of the case there will with every antigen be a certain number of borderland cases.

When a stock of antigen is made and laid by it will require to be re-tested from time to time.

6. The indicator antigen.

The red blood corpuscles which supply the indicator antigen are obtained by centrifugalising whipped or decalcified blood. The deposit of corpuscles is then thoroughly washed in physiological salt solution, and the corpuscles finally come into use in the form of a 10 per cent. suspension in physiological salt solution.

Such a suspension will keep for several days in the ice-chest; but it is essential, in every deflection of complement test, to put up a control to make sure that the corpuscular suspension which is employed shows no trace of "spontaneous" hæmolysis.

Very many different kinds of red corpuscles have been used, in particular those of man, the rabbit, the ox and the sheep.

We employ sheeps' corpuscles when we use for breaking up the indicator antigen the hæmolytic substances which are native to human serum.

There is, on the question of the propriety of relying upon these hæmolytic substances, much difference of opinion.

In favour of making use of them there is the great consideration of convenience; and there is clearly also, on general grounds,

an advantage from the point of view that every procedure should be as simple as possible, so as to avoid the fallacies which are prone to creep into all complicated techniques.

But these considerations are by some workers held to be counterbalanced and more than counterbalanced (*a*) by the consideration that in a certain small percentage of human bloods the natural hæmolytic power for sheeps' corpuscles makes default, and (*b*) by the consideration that if we use the complement and immune body for sheeps' corpuscles, which are contained in the particular bloods which we are testing, we are operating with unmeasured and varying quantities of immune body and complement, when we might, by decomplementing our bloods and adding immune body and complement separately, be operating with measured and equal quantities of these reagents.

It will be manifest that our decision in this matter of technique ought to hinge upon the question as to whether we have here really practical or merely academical objections. In other words it ought to hinge, so far as the former objection is concerned, upon the percentage of cases in which the hæmolytic power for sheeps' corpuscles makes default in human serum, and upon the amount of additional labour involved in dealing with these cases; and it ought, so far as the latter objection is concerned, to hinge upon the limits within which the immune body and complement vary in human blood, and the amount of disturbance which this variation occasions in the working of the test.

7. Sera which are required where we decide not to use sheeps' red corpuscles as our indicator antigen.

Where we decide not to avail ourselves of the hæmolytic substances for sheeps' corpuscles which are native to human serum,we must use for our indicator antigen the red corpuscles of some other animal, and we must, as we have seen, furnish ourselves with (*e*), a serum which contains an immune body which is antitropic to the particular variety of red corpuscles which we have elected to use, and with (*f*), a serum which—while containing no immune body to those red corpuscles—contains complement.

The former can readily be prepared by immunising a laboratory animal against the particular red blood corpuscles which are in question, drawing off its blood, separating off the serum, and heating this to 56°C. to destroy the complement. Bacterial contamination apart such serum will keep almost indefinitely.

Any freshly drawn serum which does not contain an immune body antitropic to the indicator antigen, may be employed for furnishing complement. Guinea-pigs' blood as being richest in complement, is most frequently used.

8. Working instructions.

To the reader who has familiarised himself with the use of capillary tubes and the diluting pipette the dilution of, and the measuring off of, the required volumes of the various reagents, will present no difficulties. These matters, therefore, may be passed over.

It may, however, be well by way of a paradigm, briefly to set out the procedure for conducting a Wassermann's test, and I follow here the method devised by my fellow-worker, Dr. Alexander Fleming.

It may be premised that the indicator antigen here employed consists of sheeps' corpuscles, and that we avail ourselves for the purpose of breaking it down of the hæmolytic substances which are native to human blood.

And we may assume that in addition to the unknown blood which is to be tested we have at our disposal two control bloods —one being a known syphilitic blood, and the other a known normal blood.

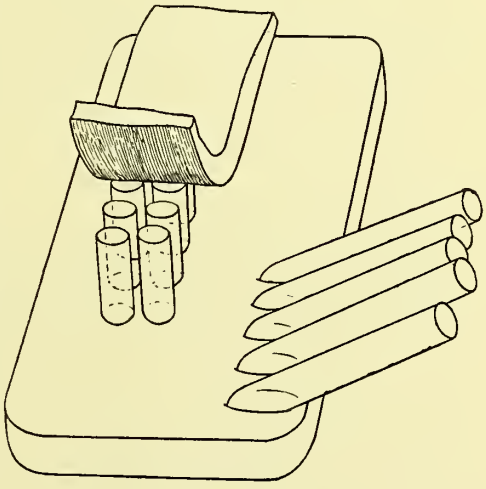


Fig. 65.

We begin by arranging our Lilliputian test-tubes in our plasticine tray in four rows of two tubules each, as shown on the left-hand side in Fig. 65. The two tubes in the first row are assigned to the controls of our materials: to the control, which certifies us that the corpuscular suspension does not show any sign of spontaneous hæmolysis, and to the control which certifies us that the antigen does not hæmolyse the corpuscles. The two tubules in the second row are for the known syphilitic blood; those in the third row for the unknown blood; and those in the fourth row for the known normal blood. And in each of our four rows we may reserve the left-hand tubules for the actual deflection of complement test, and the right-hand tubule for the control which certifies us that the serum which is being tested possesses the power of hæmolysing sheeps' corpuscles.

In a first operation we introduce into *Tubules* 1 *and* 2 of each of three nearer rows, a convenient amount of serum, let us say 10 cmm., employing in *Row* 2 the known syphilitic serum, in *Row* 3 the unknown serum, and in *Row* 4 the known normal serum. We

then add to *Tubules No.* 1 in each of the four rows the quantum of lipoid extract which our standardising experiments have shown to be appropriate, previously diluting down such extract so that the percentage of alcohol in the tubules shall not exceed 5 per cent. This done we add to *Tubules No.* 2 in each of the four rows as much 0·85 per cent. NaCl solution as will give us everywhere the same volume of fluid.

The contents of the entire set of tubes are now mixed by raising the plasticine tray from the bench, and inverting and re-inverting it several times in succession. This done, we take a slab of plasticine and bring it down upon the tops of the tubules, as shown in Fig. 65, making in this way an efficient air-tight cover. The plasticine tray with the covered-in tubules is then placed in the incubator.

After an interval of half an hour to an hour we take it out of the incubator, raise the plasticine cover, and now add to each of the eight tubules a convenient amount—let us say 10 cmm.—of a 10 per cent. suspension of sheep's corpuscles, to serve as an indicator antigen; mix by the same procedure as before; and then cover in and replace in the incubator for another half-hour.

We then read off the results.

9. Method of reading off and interpreting the results.

We look first to the rows and tubules, in which we can, if everything has gone right, foresee the results. These ought to correspond to those set out in the table as printed below.

	Tubule No. 1 (Lipoid extract and corpuscles).	Tubule No. 2 (Salt solution and corpuscles).
Row 1.—Controls of the antigens apart from serum -	no hæmolysis	no hæmolysis
Row 2.—Known syphilitic serum -	no hæmolysis	hæmolysis
Row 3.—Serum under investigation	—	hæmolysis
Row 4.—Known normal serum -	hæmolysis	hæmolysis

It will now be clear that if on turning to *Tube* 1 of *Row* 3, we find that the red corpuscles in that tubule are not hæmolysed (as in Plate II., Fig. 4, b), the blood under investigation is a syphilitic blood; while if the corpuscles are hæmolysed (as in Plate II., Fig. 3, *b*) the unknown blood is a normal blood.

A final word may be said on the significance of *hæmolysis*, or *absence of hæmolysis*, in such a record as that given above.

Where a tubule shows hæmolysis this will warrant us in inferring, either (*a*) that "spontaneous" disintegrative changes have taken place in the corpuscular suspension, or (*b*) that the lipoid extract has broken down the corpuscles, or (*c*) that complement, working in association

with an immune body which is antitropic to the corpuscles, has broken them down.

Where the alternatives (*a*) and (*b*) are excluded (as they are here by the controls in *Tubules* 2 *and* 1 of *Row* 1), we are warranted in inferring that the hæmolysis in *Tubules Nos.* 2 of *Rows* 2, 3 *and* 4, and *Tubule No.* 1 of *Row* 4 (we may leave *Tubule* 1 of *Row* 3 out of consideration) is due to complement working in association with an immune body which is antitropic to our indicator antigen; and we may therefore conclude, in the case of Tubule 1 of Row 4, that the complement has not been deflected by the differentiating "syphilitic" antigen; and from this that this blood is not syphilitic.

Where (as in *Tubule* 1 of *Row* 2) a tubule which contains serum shows no hæmolysis, and when, as here, proof is afforded (by Tubule 2 of Row 2) that both immune body and complement were originally present in the serum, we are warranted in inferring that the complement has been removed by digestion with the differentiating "syphilitic" antigen; and from this that the blood is syphilitic.

CHAPTER XI.

TECHNIQUE WHICH FINDS ITS APPLICATION IN CONNEXION WITH VACCINE-THERAPY.

1. Introductory.

The technique which we have to deal with in this Chapter may conveniently be distributed into two sections : the first dealing with the *preparation of vaccines* ; the second with the *method of handling these.*

SECTION I.—METHOD OF PREPARING VACCINES.

2. Preliminary elucidation.

A *vaccine* may be defined as a chemical agent which, when introduced into the body, elicits by a sort of chemical reflex action, an elaboration of substances which have the property of fastening upon it—neutralising it, or disintegrating it. The *bacterial vaccines*, which we have here more particularly in view, are sterilised and enumerated suspensions of bacteria which furnish when they dissolve in the body substances which stimulate the healthy tissues to a production of

specific bacteriotropic substances, which fasten upon, and directly or indirectly contribute to the destruction of, the corresponding bacteria.

3. Principle of the method which is employed.

Vaccines are manufactured by preparing pure cultures of the bacteria which are to be attacked; making suspensions of these; enumerating them; sterilising them; and then making the appropriate dilutions in weak solutions of antiseptics.

The technique for carrying out certain of the steps in the procedure differs according as we are making small quantities of a vaccine for the use of an individual patient, or a large quantity for employment as a stock vaccine.

4. Question as to when an "autogenous" and when a "stock" vaccine ought to be employed.

An "*autogenous*" vaccine—*i.e.*, a vaccine which has been made from cultures of the offending microbe obtained directly from the patient—manifestly gives us the best guarantee that the bacteriotropic substances generated in response to it will be such as to enter into destructive combination with the microbe which is responsible for the infection.

Where we employ a stock vaccine—*i.e.*, a vaccine made from the same species of microbe as the infecting microbe, but not from cultures which are directly affiliated to it—our assurance with regard to the utility of the vaccine will be based upon the fact of its having been made from a microbe which is closely conformable to that which is responsible for the infection.

The answer to the question as to whether this latter guarantee is adequate, and under what conditions it is adequate, will emerge when we have found an answer to the following series of questions:—

(*a*) *Do such minor differenees as subsist between different cultures of the same species of mierobe eome into aeeount in eonnexion with the effieacy of a vaeeine?*—Up to the present no experimental method has been devised which is capable of telling us with any certainty which of two varieties of a vaccine will be the more efficient. As matters stand, if we were required to determine such a point it could only be done by working out immunisation curves for a duplicate series of groups of men or animals, each of such groups being inoculated with a different dose of one or other variety of vaccine, but the doses being the same for corresponding groups in either series. There would still, however, if this were done, remain the possibility that the relative position of the two vaccines in the scale of merit might have

been reversed if a larger range had been given to the experiment, and if comparison had been instituted, not only between the vaccines employed in equal quantities, but also between these employed in unequal quantities.

We must, therefore, here fall back for guidance upon impressions derived from clinical experience.

Now these are quite definite on the point that a microbe which has been converted into a vaccine soon after it has been isolated from the body furnishes in general a more efficacious vaccine than a microbe which has been long subcultured on artificial media.

Where, therefore, we have only a choice between an autogenous vaccine—which will almost certainly have been made from a freshly isolated culture—and a stock vaccine which has been made from the same species after long subculturing, we shall be well advised always to elect for the autogenous microbe.

In the case, however, where we have a choice between the autogenous vaccine and a stock vaccine made up from a freshly isolated culture the advantage in favour of the autogenous vaccine will not be anything like so conspicuous. Here there will even be cases where the advantage may appear to lie with the stock vaccine.

And, in reality, the fact that a stock vaccine will often seem to do everything that an autogenous vaccine is capable of doing does not afford legitimate ground for surprise. The immunity which is conferred by prophylactic inoculations is, all of it, immunity which must be set down to the account of stock vaccines. And the science of bacteriology would not deserve to rank as a science, if it were never able to certify us that a microbe derived from one patient was fundamentally the same as a microbe derived from another.

(*b*) *Have we, when we employ an autogenous vaccine, an absolute guarantee that we are employing the actual microbe which is responsible for the patient's infection, and not merely a closely allied microbe of the same species?*—The conviction that an autogenous vaccine must—given that the bacteriological work in connexion with its making has been properly carried out—always furnish us with an absolute guarantee of the identity of the microbe in the vaccine with the microbe which is responsible for the infection may clearly count for a great deal when we are making our election between an autogenous vaccine and a stock vaccine. In reality, however, the use of the autogenous vaccine does not in every case furnish us with that guarantee. In the case, for instance, where we have to deal with periodic recurrences of boils with periods of intervening quiescence, and in particular in the case where those boils crop up indiscriminately all over the body, we cannot have any real certitude

that the microbes in the various boils are all affiliated. There is thus no justification for concluding that a vaccine made from an earlier boil will have any advantage over a stock vaccine from the point of view of its furnishing us with a microbe identical with, or even more nearly related to, that in the boils which develop later.

(*c*) *Under what circumstances may we expect to gain definite advantages from the employment of a stock vaccine?*—Consideration will make clear that we stand to gain advantages from the use of a stock vaccine in a very large percentage of the cases which present themselves for vaccine treatment—to wit, in all acute cases where it is essential to resort to treatment without delay; in all cases where, owing to the fact that the microbe is inaccessible, there would be no opportunity for making an autogenous vaccine; further, in all cases where the culture of the microbe is a matter of considerable difficulty; and, lastly, in every case where the economic condition of the patient forbids the undertaking in connexion with his case of any work which is not strictly necessary.

In all these cases the question of the possibly somewhat inferior effectiveness of the stock vaccine would fall completely out of account.

(*d*) *Have we any ready means of assuring ourselves that the microbe from which the patient is suffering is closely conformable to that which is furnished in the stock vaccine?*—It is, as will have been seen, an essential condition in connexion with the employment of stock vaccines that we should have assurance that the microbe from which the patient is suffering is closely conformable to that from which the stock vaccine was manufactured. A moment's thought will show that it is a further precondition of their usefulness that we should have at disposal quite simple means of satisfying ourselves on that point. This is the case in connexion with the majority of pathogenetic microbes. For, in the case of these, their morphological appearance and staining properties, as revealed in microscopic preparations made directly from the patient, or the appearances of the first cultures, or both of these in combination are diagnostic—to say nothing of the fact that in the case of ordinary superficial staphylococcic and streptococcic infections the clinical appearances are practically pathognomonic. In such cases a way is manifestly opened for a useful employment of stock vaccines. When, on the other hand, we have, before arriving at a bacteriological identification, to make a manifold series of subcultures, there will generally be a great saving of labour in dispensing with the refinements of bacteriological diagnosis, and setting to work immediately to make an autogenous vaccine.

5. Method of making the cultures for the preparation of a vaccine, preparing suspensions of the growth, and resolving this into separate bacterial elements.

The cultures which are to be worked up into a vaccine are best made on solid media, *first*, because these give a more rapid and copious growth; *secondly*, because the bacterial elements are less aggregated together than they would be if grown in fluid media; and, *thirdly*, because, if any of the cultures should be contaminated, this would, on solid media, be more easily detected. With a view to further diminishing the chance of contamination, it will be advisable in every case to make the vaccine, not from the original culture, but from individual colonies grown on subculture; and it will also be advisable to make zig-zag cultivations, which give separate colonies; instead of flushing the agar with a bacterial suspension to make mass cultures.

Where we are making a vaccine for an individual patient it will in the case of a very freely growing microbe, such as the staphylococcus or any member of the coliform group, suffice to plant out on two sloped agar tubes; but it will be well when dealing with microbes such as streptococci and pneumococci, which give a much less luxuriant growth, to use four to six tubes.

The cultures may usually be grown for 24 hours at 37° C, but in the case of coliform organism a much shorter period in the incubator will amply suffice; and where it is a question of great urgency, it will generally, even in the case where we are dealing with a streptococcus, be possible to get enough material to make up a vaccine in six to eight hours.

When the cultures are ready a sample of the growth is taken from each, and these are stained and examined under the microscope to see that we have in hand cultures of the right organism, and pure cultures.

This point settled, we take a test tube of sterile physiological salt solution, and—working throughout with aseptic precautions—pour a portion of it over the surface of the first of our cultures, shaking up the fluid in such a way as to bring the microbes into suspension. We take a platinum loop to our aid, if the microbes do not come off easily from the agar, but if we do so, we must not cut into the agar and mix up fragments of this with our bacterial suspension.

We pour the bacterial suspension thus obtained on to the surface of our second culture, bring this also into suspension, and repeat this process adding more salt solution if necessary as we proceed, till we have gone through our whole series of cultures. This done, we

transfer the final suspension to an empty sterilised test-tube, and seal this off in the manner described in *Chapter II., Sub-section* 11, *a*.

If we have not now at disposal an absolutely even suspension consisting of isolated bacterial elements, we must endeavour to break up the bacterial masses, or, failing that, to get these out of the way.

An efficient mechanical shaker such as that designed by my fellow-worker, Dr. John Matthews (Fig. 66), and an effective centrifuge (*vide Chapter I., Sub-section* 10) will be required for the purpose.

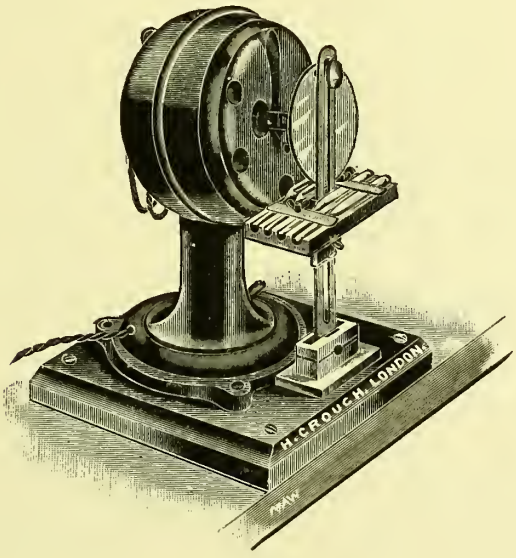


Fig. 66.

In employing the mechanical shaker, the sealed test-tube containing the bacterial suspension is firmly clamped down on the shelf of the machine. If, after 5 to 10 minutes, no progress has been made—and this will not unfrequently be the event where we are dealing with diphtheroid microbes—it will be well to try the effect of warming the suspension, and also the effect of rendering it slightly alkaline by an addition of sodium bicarbonate.

If it is a question not of the whole of the suspension consisting of aggregated masses of bacteria, but only of a few clumps remaining behind in an otherwise even suspension, these may be removed either by allowing them to sediment or by a very short centrifugalisation, the supernatant emulsion being afterwards siphoned off into a siphon-test-tube—made by drawing out the lower end of a test tube into a stem,

bending this round into a horse-shoe loop, and then bending the long thin limb of the horse-shoe round, as in making a siphon capsule (*Chapter III., Sub-section* 12), so as to give us the siphon-test-tube shown in Fig. 67.

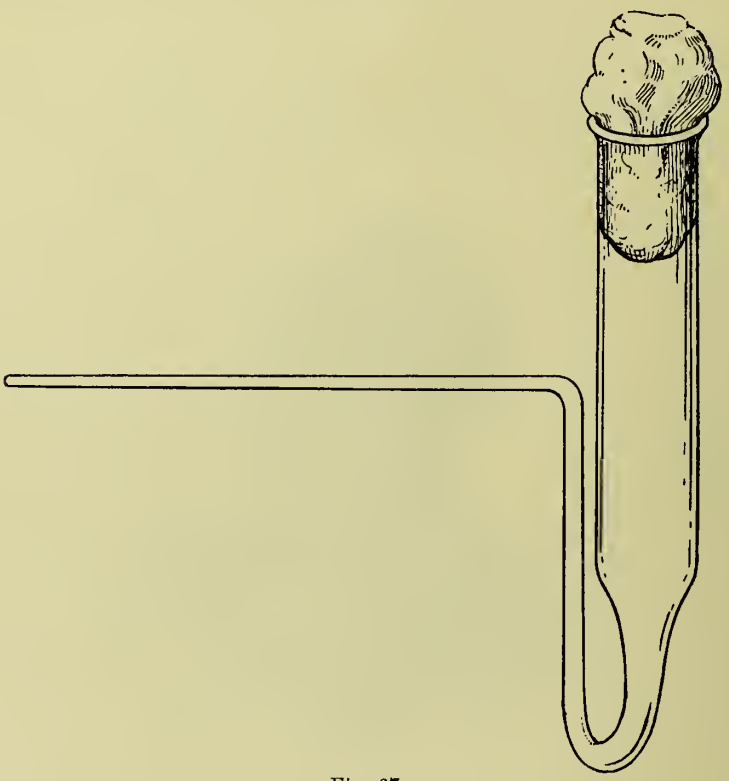


Fig. 67.

6. Method of enumerating the bacterial elements contained in the unit-volume of the suspension which is to serve as a vaccine.

The enumerating of the bacterial elements in a vaccine is undertaken because it enables us, where there is previous experience of inoculations with a similar microbe, to adjust our initial dose in accordance with that experience, and to turn to account in each subsequent case the experience which we are accumulating from the past.

In dealing with a vaccine we have to enumerate both dead and living bacteria without distinction, for both alike will furnish when dissolved the toxic chemical agent which calls forth the elaboration of bacteriotropic substances. We can do this only by counting a sample of the suspension under the microscope, and inasmuch as sharp

definition and the staining properties of the microbes are often lost in the process of sterilisation by heat the sample of vaccine is taken for enumeration before that sterilisation is undertaken.

The problem of counting the number of microbes in the bacterial suspension is solved by mixing with a measured volume of the suspension a measured volume of blood drawn from the finger.

Then seeing that we know the number of red blood corpuscles contained in a standard volume of normal blood, and since we mix the blood and the bacterial suspension in measured volumes, all that we shall require to arrive at the number of bacteria in 1 cc., or any other standard volume, of our suspension will be to elicit by sampling the proportion of corpuscles to bacteria in our mixture.

The actual steps we have to take are as follows :—

We pass the stem of our vaccine tube through the flame in order to sterilise its outside ; then, when it has cooled down again, shake up the contents of the tube so as to mix thoroughly ; and then proceed to shake down a portion of the contents—perhaps a tenth of a cubic centimetre, or a little more—into the distal end of the stem. We now make a nick with a glass-cutting knife a little beyond the line to which this fluid reaches, and then snap across the stem—obtaining in this way a tubule containing a sample of our suspension. We set this tubule upright in our plasticine tray, re-seal our test-tube, set it aside for subsequent sterilisation, and commence the measuring operations for our count.

We begin by taking a simple capillary pipette, making a mark upon its stem and fitting a teat to the barrel. Having laid this ready to hand, we now prick into our finger, press out a drop of blood, and then taking up our pipette, draw up into it, first, one volume of blood and then either one volume of our bacterial suspension; or two or more volumes, if it appears to us on critical inspection—and the capacity for judging approximately by naked eye inspection of the number of microbes in a suspension is soon acquired—that it contains very much less than 5,000 millions of microbes in the cubic centimetre.

We now have everything that is really essential ; but it will be well to guard against a possible crimping of the red corpuscles in the drying of our films. Such crimping may be avoided if, after aspirating into our pipette the unit-volumes of blood and bacterial suspension, we follow on with one—or in the case where we use more than one unit-volume of bacterial suspension—two volumes of distilled water.

We now expel from our pipette first only the distilled water and the bacterial suspension, and mix these—so that there may be no danger of the red corpuscles being hæmolysed—and then proceed to

mix together the whole contents of our pipette, aspirating and re-expelling these some dozen times.

This done, we make from our mixture two or three microscopic films, spreading these out with an ordinary slide used as a spreader, upon slides which have been roughened with emery (*Chapter VI., Sub-section* 3).

When dry the films are to be fixed and stained by pouring over them a saturated solution of corrosive sublimate, washing this off thoroughly, and staining with carbol-thionin ; or they may be simply fixed and mounted in water.

The films may now be subjected to a preliminary scrutiny. If we find red blood corpuscles and microbes in approximately the same numbers, and find our suspension free from bacterial aggregates we may proceed with our count. If either the bacteria or the corpuscles are in large excess we must make new mixtures and new films—using either more than one unit-volume of blood to one unit-volume of bacterial suspension, or, as the case may be, more unit-volumes of bacterial suspension. If we find that we have on our films a number of bacterial aggregates, we must take our bacterial suspension in hand again and try to break it up more effectually. But we may note in passing that if we fail in this, and have to give up all attempts at counting, we can still make use of our vaccine, proceeding then in our dosage entirely by trial and error as in the case of an altogether untried vaccine.

When satisfactory films are obtained we pass to the actual counting. This is carried out with an oil immersion lens and the highest available eyepiece so as to restrict the field of view and bring down the numbers of elements in the microscopic field to a number that can be conveniently enumerated. Failing a sufficiently high eyepiece we inscribe cross lines on a small coverglass or a segment of a coverglass, and drop it on to the diaphragm of the eyepiece. In the case where we use an unstained preparation the illumination will of course be adjusted in such a manner as to bring both the bacteria and corpuscles plainly into view.

Everything being now arranged we choose out a microscopic field at random and, taking pencil in hand, note down on a sheet of paper, or upon the tiles of the laboratory bench, the number of red corpuscles and the number of bacteria encountered—enumerating these separately, and writing the numbers down in two columns. Proceeding in this way, we pass at random from field to field, traversing every region of the slide, and applying the same code of morality as in the case of the opsonic film. That is to say, we come to some understanding with ourselves as to how the corpuscles which

transgress or touch the edge of the field are to be counted (we may conveniently count these alternately in and out); we eliminate from consideration any regions of the film where the preparation is unsatisfactory with respect to staining or with respect to the integrity of the red corpuscles; and we go on counting until we find that the counting of one or two additional fields ceases to make a difference to the result.

Finally, when we find that we have counted enough, we set out our results in the form of a sum in compound proportion :—

No. of unit vols. of bacterial suspension : No. of unit-vols. of blood : :
Number of red corpuscles counted - : number of microbes counted
Number of red corpuscles in 1 cc. of blood (*i.e.*, 5,000 million):
Answer.—Number of micobes in 1 cc. of the bacterial suspension.

Example.—1 volume of blood has been mixed with 3 volumes of bacterial suspension, and the numbers of red blood corpuscles and bacteria counted are respectively 455 and 397.

3 : 1 : : 5,000 million : *Answer.*—1,500 million, nearly, in 1 cc.
455 : 397

7. Method of sterilising the vaccine and testing its sterility.

When the count has been completed, or during any pause in the proceedings, we set about the sterilisation of the vaccine. We may either heat it in a water-bath or add an antiseptic.

If we adopt the former method—and it is probably the best, and, with a view to the after-coming tests for sterility, the simplest—we introduce our vaccine into the water-bath. It is convenient to use a water-bath provided with a regulator such as is shown in Fig. 68; and we may also—with a view to keeping the whole of the vaccine tube immersed—appropriately employ some such form of holder as that designed by my fellow-worker, Dr. Alexander Fleming (Fig. 69). In default of such a holder we may put a funnel down over the vaccine or roll it round with a sheet of lead which has been perforated by a string to facilitate the withdrawal of the tube from the hot water.

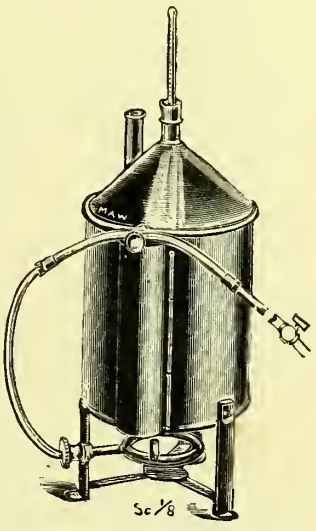


Fig. 68.

With the water-bath standing at 55° to 60° C, sterilisation will nearly always be complete at the expiration of twenty minutes. But in the case of the staphylococcus it will be well to prolong the heating to one hour. And Leishman has demonstrated that it is in the case of typhoid vaccine advisable that the temperature shall not exceed 55° C.

When a vaccine is very urgently required we may take the efficacy of the above heat sterilisation on trust, and proceed immediately to dilute and use the vaccine. But in all other cases it will be proper to assure ourselves of the efficacy of our heating by making cultures. For it is always a possibility that contaminating microbes—and in particular spore-bearing microbes, such as the bacillus subtilis and the bacillus mesentericus — may have found an entrance into our vaccine, and may not have been killed off in the waterbath.

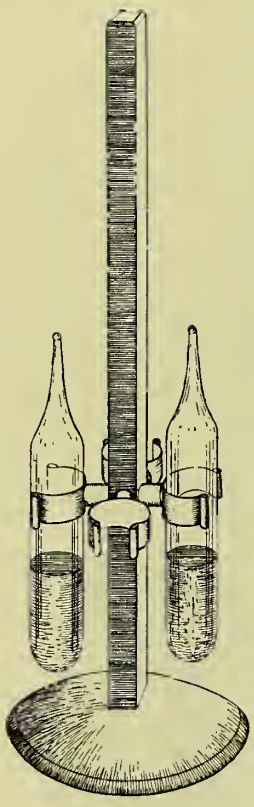


Fig. 69.

We make these test-cultivations by breaking off the tip of our vaccine tube, inverting it, introducing the open nozzle into the mouth of an agar tube, and then bringing the flame of a Bunsen to bear upon the bottom of the vaccine tube in such a manner as to heat the contained air, and blow out a drop or two of the vaccine on to the surface of the agar slope.

This last is now placed in the incubator to be inspected after the lapse of twenty-four hours, and the vaccine tube is sealed up and put away, to await the result of the test—name, date, and number of microbes per cc. being first inscribed upon the tube with a glass-writing pencil.

Before, however, the vaccine is sealed up to be put by, it may advisably receive a measured addition of an antiseptic ($\frac{1}{4}$% of lysol or $\frac{1}{2}$% of carbolic acid) as a preservative against chance contamination. We here proceed as follows:—We first roughly gauge the volume of our vaccine by measuring with a centimetre rule the height of the column of vaccine and the diameter of the test-tube. We compute the cubic contents of this cylinder of fluid by multiplying its length by its diameter squared, and the result by 0·78. Having arrived in this way at the volume, we take a pipette which has been graduated in terms of cubic millimetres, or an automatic pipette, and draw up into it the quantity of undiluted antiseptic which is required. We now warm the neck of our open vaccine tube, and, as soon as it begins to cool again, insert the point of our pipette, blow out our antiseptic, and let the indraft of air carry it into the interior of the test-tube.

8. Method of diluting and putting up vaccine bottles, and of filling out single doses into vaccine bulbs.

When the sterility of the vaccine is assured we consider what will be the proper dilution to give us within the compass of 1 cc. a range of doses such as will meet the probable requirements of the patient.

To make that dilution, we provide ourselves with rubber-capped bottles, such as are shown in Fig. 72. These are got ready for use by sterilising them, filling them in with 25 cc. of a $\frac{1}{2}$% carbolic and 0·85% NaCl solution, and then capping them with sterile rubber caps (Fig. 70), which are fastened down on their seat by a couple of turns of brass wire, and are finally coated over with a layer of hard paraffin. We take one or more of such bottles, sterilise the surface of the rubber by transferring to its surface a drop of undiluted lysol, and then — turning the bottle bottom upwards—puncture through the rubber with a comparatively fine needle fitted to a sterilised syringe. We now withdraw such volume of fluid as will make room within our 25 cc., for the quantum of the original vaccine, which will give us, when diluted in 25 cc., a vaccine of the strength we require.

Fig. 70.

We have now to introduce that quantum into the bottle. We do so in the following way. Having first emptied our syringe, we break off the tip of our vaccine tube, and then, grasping it by the neck, and not by the base where the warmth of our

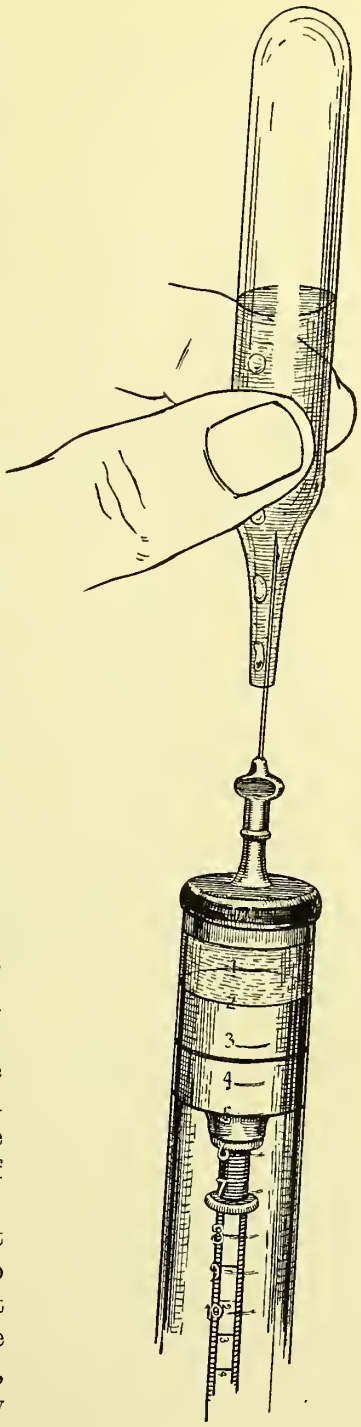


Fig. 71.

fingers would heat the contained air, we invert the test tube, and then holding it slightly on the slant as in Fig. 71, we introduce the syringe into its open end, keeping the needle along the lower wall, and the eye of the needle turned towards this—so as to allow the bubbles of air which enter the tube, as we withdraw the vaccine, to pass free.

Fig. 72.

Having withdrawn from the tube such quantum as we require, we introduce this into the bottle by puncturing as before through the cap, and then label our bottle as shown in Fig. 72.

EXAMPLE 1.—Required a 10-fold dilution of our original vaccine. We withdraw 2·5 cc. of carbolised salt solution from the 25 cc. in the bottle and replace this by 2·5 cc. of our original vaccine.

EXAMPLE 2.—Required a 500-fold dilution of our original vaccine. We here take two 25 cc. bottles, withdraw from the first 1 cc., and from the second 1·25 cc. of carbolised salt solution; then transfer 1 cc. of the original vaccine to the first bottle; shake up carefully and then transfer 1·25 cc. from Bottle 1 to Bottle 2.

Where we want to fill in a single dose, or a series of single doses of vaccine, into vaccine bulbs (Fig. 73) we open up a series of capsules, nicking them and breaking across their capillary stems after passing these through the flame. We then fill in the required doses, withdrawing vaccine from the bottle with a syringe and measuring out with this into the bulbs. We may then, if the volume of the dose is too small to handle with accuracy, fill up with an unmeasured addition of sterilised carbolised salt solution. Finally we seal the bulbs as explained in *Chapter II., Sub-section* 11, *b*.


Fig. 73.

9.—Special technique which comes into application where stock vaccines in large quantities are prepared.

Where large quantities of stock vaccine are required the technique which has been described above as modified in the following particulars:

(*a*) *Method of making the bacterial cultures.*—We take for the bacterial cultures, not agar sloped in the ordinary way in test-tubes, but agar spread out flat over the floor of large triangular bottles (Roux's bottles). The mouth of these is covered in with a special stout rubber cap, which runs out into a rubber tube, and is firmly tied down upon the neck with a knot whose four loose ends

go round the base of the bottle. The end of the outlet tube is fitted air-tight into a piece of glass tubing which at the outset is plugged with cotton wool, and which serves afterwards for establishing a connexion with a further piece of apparatus. The whole is sterilised in the autoclave. When a cultivation is to be made on such a bottle we take a sterilised 5 to 10 cc. syringe fitted with a moderate-sized needle, fill it in with a suspension of the microbe we require, sterilise the surface of the rubber cap, or of the outlet tube, by touching it with the point of a heated glass rod, and piercing it at this spot with the needle inject

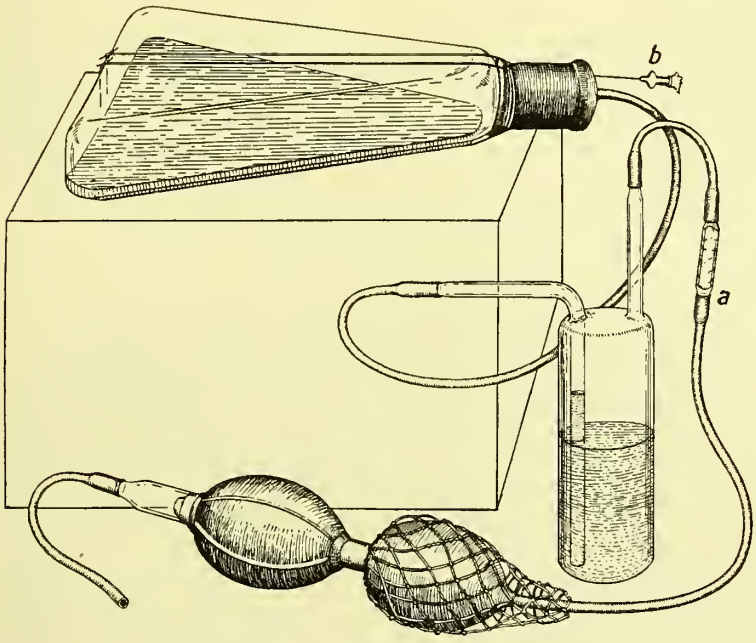


Fig. 74.

the contents of the syringe into the bottle. We now rock the bottle from side to side so as to flush the whole service with the suspension, and then before placing the bottle in the incubator, again take a heated glass rod and apply it to the surface in such a way as to melt a little of the india-rubber and close in the puncture. This done we place the bottle in the incubator—disposing it so that the agar film may be on the roof and not on the floor of the bottle. This is done, not only to drain off the excess of bacterial suspension, but also to prevent the condensation water flooding the surface of the agar.

(*b*) *Method of harvesting the microbial growth.*—The apparatus used for this purpose and the method of procedure will be elucidated by Fig. 74.

We have here our Roux's bottle, which contains the bacterial growth. It has, as will be seen, been connected up by means of its outlet tube with a wash bottle containing fluid, to which is attached a rubber ball pump. The fluid in the wash bottle is sterilised physiological salt solution. An outlet from the Roux's bottle is provided by puncturing the rubber cap with a hypodermic needle, and this outlet,

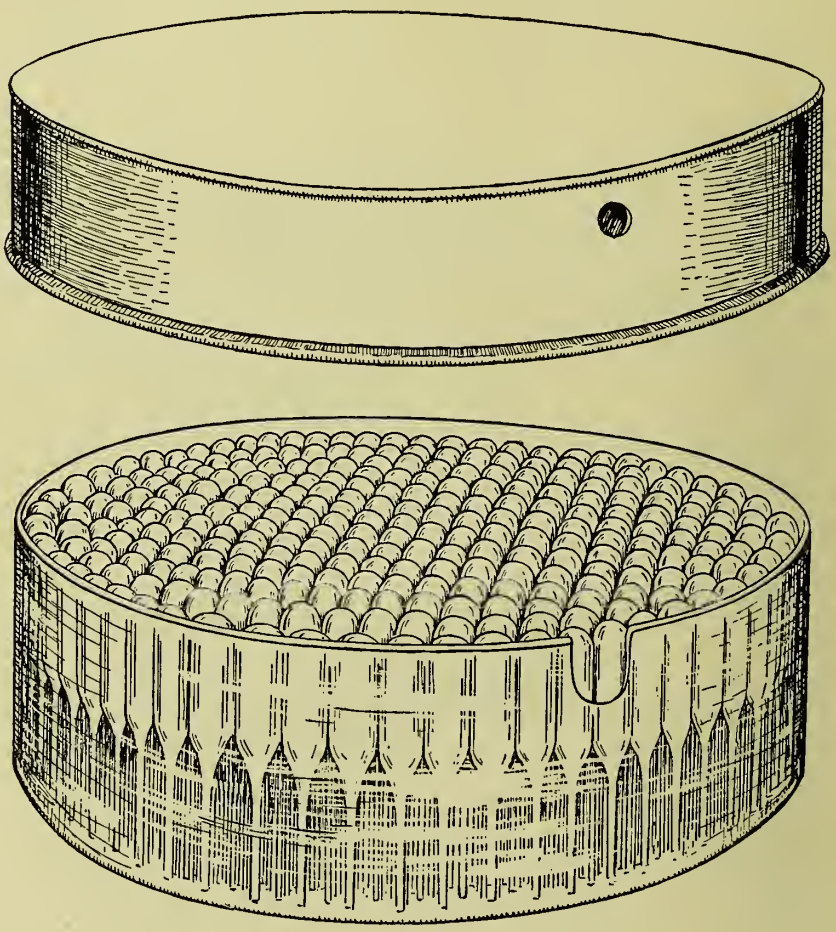


Fig. 75.

as well as the connexion between the wash bottle and the rubber ball pump, is safeguarded by a plug of cotton wool.

The method of procedure is as follows:—The sterilised salt solution is driven over into the Roux bottle by working the pump, and the suspension is then made by rocking and shaking the bottle. We now unship the rubber bellows, and join it up with the wash bottle in the reverse manner—so as to make it work as a suction pump. With this

object the butt end of the rubber pump which carries the valve has been fitted airtight into the mouth of a narrow test-tube, which has been drawn out below in such a manner as to fit the rubber tube which makes the connexion.

When the suspension has in this way been aspirated into the wash bottle a sample is withdrawn for counting, and the two arms are sealed in the blowpipe flame, as a preliminary to immersing it in the water bath for the sterilisation of the vaccine.

The principle of the method which is here in question was suggested by my fellow-worker, the late Dr. J. H. Wells; the final working out is due to Captain S. R. Douglas.

(*c*) *Method of filling in a batch of vaccine bulbs simultaneously.*—The method which is employed is elucidated by Fig. 75. We have here a batch of vaccine bulbs packed closely together into a glass dish, which is provided with a valvular lateral opening, which can be opened and closed by turning round the cover. The vaccine bulbs are turned upside down, and their ends are, as will be seen, open. The apparatus thus filled in is sterilised in the autoclave. When it is to be brought into use we turn the lid round, so as to open up the inlet, and introduce through this sufficient vaccine to provide for each bulb the quantum allotted to it, and whatever additional allowance may be found necessary to secure that no individual bulb shall obtain less than its due owing to another getting more than its share. As a further precaution against such an accident, it will be well to eliminate all bulbs that are above standard size, and to see that the floor of the dish is quite level, so that so long as there is any vaccine in the dish it shall be equally available for all.

The filling is done by placing the dish under the bell of an air-pump and exhausting the air, and then re-admitting it.

After the bulbs have been sealed in the blow-pipe flame, they are packed into the dish in the same manner as before, and again placed under the air-pump. It is clear that if there is any bulb which has not been properly sealed it will now be emptied.

The procedure here described was adapted for use in connexion with vaccines by my fellow-worker, Dr. L. Noon, the device for testing the efficacy of the sealing being afterwards added by Dr. J. Freeman.

SECTION II.—METHOD OF HANDLING AND INOCULATING THE COMPLETED VACCINE.

10. Choice of a syringe.

The syringes which are best adapted for all work in connexion with vaccine-therapy are syringes which have a metallic plunger and a glass barrel fixed in by a special metallic solder. These are made in all sizes

up to 100 cc. or more. For the actual injection of vaccine the 1 cc. size is all that we require, but the 5, 10 and 20 cc. sizes find all sorts of useful application in connexion with the manufacture and dilution of vaccines.

11. Method of sterilising syringes.

Syringes may of course be sterilised by prolonged boiling in water. Trouble and time may, however, be saved by employing for sterilisation, a temperature higher than the boiling point of water. We can obtain this from heated oil, and a temperature of 125° to 140° C. will sterilise instantaneously everything with which it comes into contact. If the oil is not heated above 140° C., it can be drawn into the syringe without affecting the solder, and there would be no danger to the glass even if the oil had reached a very much higher temperature.

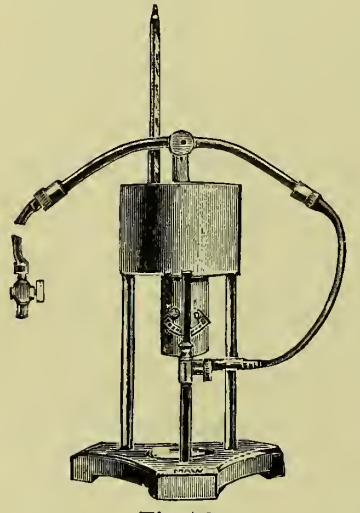


Fig. 76.

The most convenient arrangement for sterilisation both for the laboratory and out-patient and consulting-room, is to employ an oil bath provided with a regulator which will keep the oil constantly at the required temperature (Fig. 76).*

Failing an oil bath and a thermometer which registers temperatures above 100° C.—and we have in this section to take into account also technique which is applicable outside a laboratory—we may proceed as follows, using a piece of bread crumb as a thermometer to tell us when the oil has reached the required temperature.

Partly fill a tablespoon with any vegetable oil. Introduce into the oil a bread crumb about the size of a large hemp seed. Then heat over a spirit lamp until bubbles of steam begin to come off from the bread crumb. The temperature of the oil is now about that of boiling water. Wait until the bubbles have finished coming from the bread crumb—taking an opportunity meanwhile to draw up the oil once or twice into the syringe—and then very cautiously re-apply the heat until the bread crumb shows the first sign of turning brown (about 140° C.). Then allowing no time for the oil to cool down, draw it up two or three times into the syringe, taking care that it comes into contact with every part of the interior.

* Such oil-baths can be obtained from Messrs. Hearson, Regent Street, London, or from any of the laboratory purveyors whose addresses have been given (page 7).

If it be desired—on the ground of appearances—to get rid of all remaining traces of oil from the syringe, this can be very simply effected by drawing up into the syringe a very weak ($\frac{1}{4}$ to $\frac{1}{2}$%) solution of sterilised carbonate of soda.

12. Method of carrying a sterilized syringe from place to place in an aseptic manner.

The 1 cc. syringe, which is employed for inoculating, can easily be carried about asceptically in the manner illustrated in Fig. 77. Taking an empty sterilised test-tube, we sterilise the cotton wool plug by setting it alight, thrust the needle of our syringe into it, then driving it down into the body of the test-tube, as shown in the figure. The test-tube must then be carried about upright.

Fig. 77.

Where we are dealing with a larger syringe, and where a correspondingly larger test-tube is not available, we take a piece of narrow glass tubing, which will just fit over the needle, pack it full with cotton wool, sterilise it by passing it through the flame until the wool just begins to char, and then thrust our needle into it.

13. Method of drawing vaccine off from the rubber-capped bottles and bulbs, and of making any further dilutions where these are required.

(*a*) *Method of drawing off vaccine from the rubber-capped bottles and care of the bottled vaccine.*—The method for drawing vaccine off from the bottles is, of course, exactly the same as that described (*supra*, *Sub-section* 8) in connexion with the dilution of the original vaccine.

The following details, which have reference to the procedure, or to the care of the bottled vaccine, may, however, be noted:—

If the needle gets choked with the paraffin which coats the rubber-cap, and will not draw when it gets into the bottle, the obstruction may be very readily got rid of by dipping the point of the needle into the hot oil.

The negative pressure which develops in the interior of the bottle, as the vaccine is withdrawn, may be neglected until it is found that the rubber-cap is drawn in. When this happens it will be well to restore the balance of pressure by filling in our syringe with air, and injecting this into the bottle whenever we are withdrawing vaccine.

If through use of too large a needle, or through placing the needle pricks too close together, a leak should develop in the cap, this may be repaired by washing off the lysol with alcohol, and then

applying a little of the rubber solution which is used for mending bicycle tyres.

Where a bottle of vaccine will not be required again for a considerable time it will be well, as a precaution against the perishing of the rubber, to recoat it with paraffin.

(*b*) *Method of drawing off vaccine from a vaccine bulb.*—After shaking up the contents, and sterilising the tip of the stem in the flame, nick this with a glass-cutting knife and break it across; or use for this purpose a pair of sterilised forceps, or the sterilised barrel of a small key; or, in default of these, crack the glass stem by gently shaking down into it, when it is hot, a drop of the vaccine. The bulb is now inverted over the needle exactly as the vaccine tube is in Fig. 71. The same precautions are taken as are there enjoined, and as the vaccine is drawn off the point of the needle is gradually lowered so as to keep its orifice constantly below the surface of the fluid.

(*c*) *Method of making with extemporised means a further dilution of a vaccine.*—If we wish to administer a smaller dose of vaccine than can be accurately measured off from the particular dilution of vaccine we have at hand, we can provide ourselves with the sterilised fluid required for making the dilution, by sealing up a little water in a vaccine bulb, dropping it into the superheated oil, and keeping it there for a few moments.

14. Method of making the inoculations.

In connexion with the actual inoculation the question of sterilising the skin, and the question of the site of inoculation are the two issues which come up for consideration. The *sterilisation of the skin* can be enjoined only by way of a *counsel of perfection*, for—though staphylococci from the skin not unfrequently develop in blood cultures where blood has been drawn off from the veins with insufficient antiseptic precautions—*à priori* consideration and actual experience show that the chances of carrying microbes from the skin through on the point of the needle into the deeper tissues in sufficient numbers to count is very remote.

Where an antiseptic is employed it will conduce both to saving of time and to efficiency to employ a strong antiseptic such as undiluted lysol which will be instantaneously effective—taking care to wipe it off immediately after inoculation.

Both convenience, and such experimental work as has already been done to test the comparative efficacy of inoculations made into different tissues, point to the subcutaneous tissues as the most suitable *site for inoculations*. The best way to proceed will be to pick up a fold of skin between finger and thumb and then to carry the needle well down

into the middle of the fold. Where we are dealing with a vaccine—such as typhoid vaccine, which may produce a considerable amount of

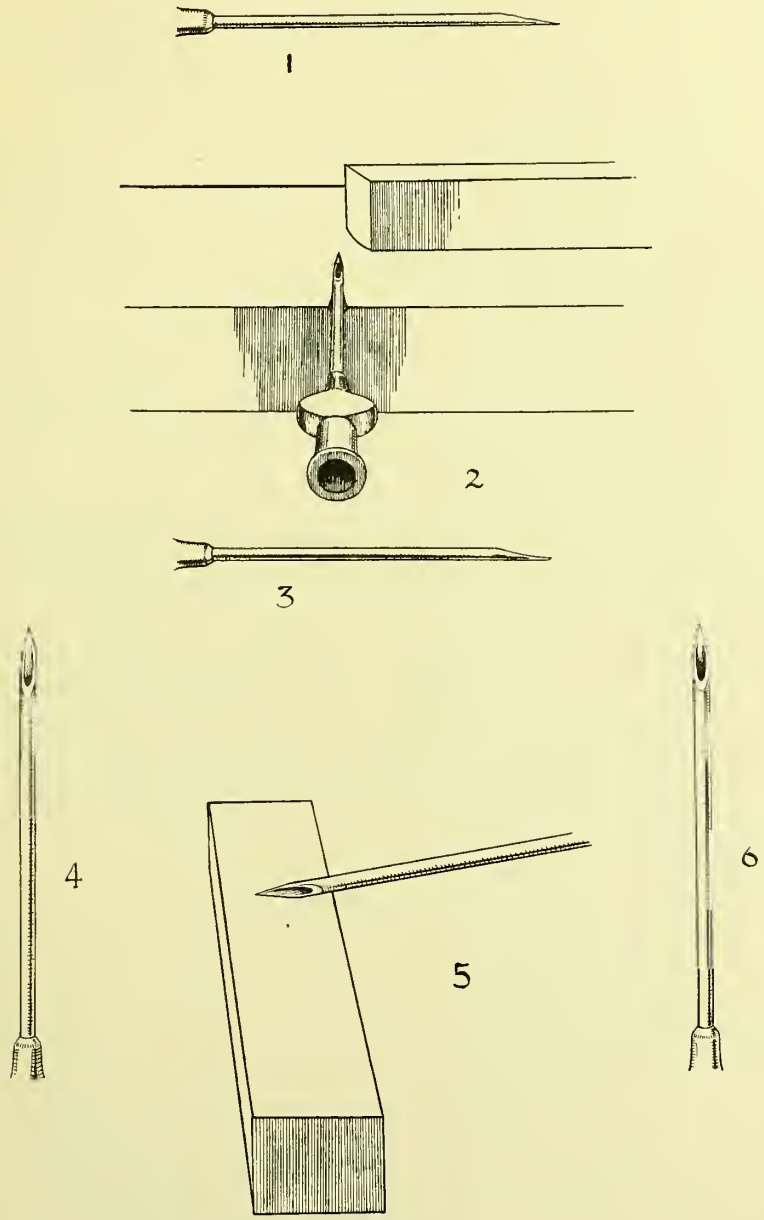


Fig. 78.

inflammatory reaction and painful swelling at the site of inoculation, and streptococcus vaccine, which also always produces some pain and

swelling—it is well to avoid inoculating into a region of the body where the skin is at all tightly bound down. The most convenient place to inoculate is then the front or back of the shoulder.

In view of the consideration that the power of response of the tissues to the stimulus of a vaccine is probably limited, it would seem advisable to choose a new site for each successive inoculation.

15.—Method of sharpening hypodermic needles.

The knowledge of how to push a needle through the skin is not all that can be required of an inoculator. He must, if he is not to discredit inoculation, know also how to put an edge and a point on to a blunt hypodermic needle. The sum total of apparatus required for this purpose is a silex sharpening stone with one slightly convex face. And practically all that is necessary may be learned from the accompanying figures. When we want to sharpen a needle we begin by cutting a groove in a block of wood, or in the edge of our working bench, in either case arranging the groove so that we can get comfortably to work upon our needle as it lies in the groove. We turn the needle with its eye pointing upwards as in Fig. 78 (2), put a drop of oil on the convex face of our sharpening stone, turn this downwards, and now grind down the face of the needle until the edge as viewed from the side tapers away gradually and evenly in a gentle curve to the point. We now put the stone down upon the bench, turn it upon its side, place a drop of oil upon it, and then, bringing down first one edge and then the other upon the plane face of the stone, grind down these edges until, on looking along them, we see that we have two bright cutting surfaces which meet together at the point.

Finally, we take a piece of fine emery paper, and polish the barrel of the needle.

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